Identification and characterization of peptide-like MHC-ligand exchange catalyst as immune response enhancer

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Content

Summary

MHC class II molecules present antigenic peptides on the cell surface for the surveillance by CD4+ T cells. To ensure that these ligands accurately reflect the content of the intracellular MHC loading compartment, a complex processing pathway has evolved that delivers only stable peptide/MHC complexes to the surface. As additional safeguard mechanism, MHC molecules quickly acquire a 'non-receptive' state once they have lost their ligand. This study shows that amino acid side chains of short peptides can bypass these safety mechanisms by triggering the reversible ligand-exchange. The catalytic activity of dipeptides such as Tyr-Arg (YR) is stereo-specific and could be enhanced by modifications addressing the conserved H-bond network near the P1 pocket of the MHC molecule. It enhanced both antigen-loading and ligand-release and strictly correlated with reported anchor preferences of P1, the specific target site for the catalytic side chain of the dipeptide. The effect was evident also in CD4+ T cell assays, where the allele-selective influence of the dipeptides translated into increased sensitivities of the antigen-specific immune response.

The hypothesis that occupation of P1 prevents the 'closure' of the 'empty' peptide binding site into the 'non-receptive' state was further supported by molecular dynamic calculations. During antigen processing and presentation P1 may therefore function as important 'sensor' for peptide-load. Spectroscopic studies using ANS dye (8-aninilino-1-napthalenesulfonic acid) and intrinsic tryptophan fluorescence data, confirm the postulate by providing direct evidence for the conformational transitions. Moreover conformation specific antibodies previously described to be specific for 'empty' MHC could be shown to be a 'probe' for 'receptive conformation'.

As potent risk factors short peptides may be involved in the induction of autoimmune diseases. It could be shown here that they could enhance the loading of gluten derived antigen on celiac disease linked-HLA-DQ2 allele. At least *in vitro* the effect could enhance gluten specific CD4+ T cell response on T cell clones obtained from celiac disease patients. Thus, on one hand short peptides might work as 'MHC loading enhancer' (MLE) in the precipitation of inflammatory-'autoimmune' disorder, on the other hand they might be used as drug like vaccine 'additive' in various therapeutic settings.

Zusammenfassung

MHC Klasse II Moleküle präsentieren Peptidantigene für die Überwachung durch CD4+ T Zellen an der Zelloberfläche. Um Sicherzustellen, dass diese Peptidliganden möglichst genau die intrazelluläre Proteinzusammensetzung widerspiegeln, hat sich im Verlauf der Evolution ein komplexer Prozessierungsweg entwickelt, welcher möglichst stabile Peptid/MHC Komplexe an die Zelloberfläche liefert. MHC Moleküle, welche ihren Liganden verloren haben, konvertieren zudem spontan in einen ‚nichtrezeptiven' Zustand, was als zusätzlicher Sicherheitsmechanismus dient. Diese Studie zeigt jedoch, dass Aminosäureseitenketten kurzer Peptide diesen Sicherheitsmechanismus umgehen können indem sie katalytisch einen reversiblen Ligandenaustausch auslösen. Die katalytische Aktivität von Dipeptiden, wie z.B. Tyr-Arg (YR), war dabei stereospezifisch und konnte durch zusätzliche Modifikationen verstärkt werden, welche das konservierte H-Brückennetzwerk der so genannten P1-Tasche des MHC Moleküls adressierten. Die Dipeptide verstärkten dabei sowohl die Antigenbeladung als auch den Ligandenaustausch, wobei deren relative Aktivität genau mit den bekanten Ankerpräferenzen der P1 Tasche korrelierte. Letzteres weist somit auf eine direkte Interaktion der katalytischen Seitenkette des Dipeptides mit dieser Tasche hin. Der Verstärkungseffekt war auch in CD4+ T Zellassays zu beobachten, bei denen der alleleselektive Einfluss der Dipeptide direkt in eine deutliche Erhöhung der Sensitivität der antigenspezifischen T Zellantwort führte.
Durch weitere molekulardynamische Berechnungen konnte die Hypothese unterstützt werden, dass die Besetzung der P1 Tasche durch Aminosäureseitenketten einen Kollaps der leeren Bindungstasche zum ‚nichtrezeptiven' Zustand verhindert. Während der Antigenpräsentation könnte P1 somit unmittelbar als ‚Sensor' für die Beladung mit Peptiden dienen. Diese Annahme konnte experimentell durch spektroskopische Untersuchungen unter Verwendung des ANS-Farbstoffes (8-Anilino-1-Naphtalensulfonsäure) sowie durch Messung der intrinsischen Tryptophanfluoreszenz bestätigt werden. Darüber hinaus konnten konformationsspezifische Antikörper, welche bislang lediglich mit unbeladenen MHC Molekülen in Verbindung gebracht wurden, hier als spezifische Sonden für den nichtrezeptiven Zustand definiert werden.
Als mögliche Risikofaktoren könnten katalytische kurze Peptide eine Rolle bei der Auslösung von Autoimmunerkrankungen spielen. In dieser Studie konnte gezeigt werden, dass sie die Beladung von Glutenantigenen auf das Zöliakie-assozierte HLA-DQ2 Molekül verstärken können. Zumindest in vitro konnte ihre Anwesenheit deshalb auch die antigenspezifische Antwort von CD4+ T Zellen verstärken, welche zuvor von Zöliakiepatienten isoliert worden waren. Auf der einen Seite könnten diese Peptide als ‚MHC-loading enhancer' (MLE) deshalb als mögliche Risikofaktoren die Ausbildung entzündlicher (Auto-) Immunerkrankungen beschleunigen. Auf der anderen Seite könnten sie jedoch auch als ‚drug-like' Vakzinadditiv zur Verbesserung von Immuntherapien führen.

1 Introduction

Immunity is defined as response against foreign substances including microbes, proteins and polysaccharides. The cells and molecules responsible for immunity constitute immune system and their combined and coordinated response to the foreign pathogens and tumor cells is termed as immune response. Important aspect in elucidation and control of immune response is the recognition of the pathogenic substance. Microbial defense is mediated by early reactions of innate or natural or native immunity and later response by adaptive immunity. Innate immunity is characterized by evolution of surface antigen receptors recognizing and binding common bacterial components. However, in adaptive immune response highly diverse and specific antigen receptors evolve by irreversible recombination and somatic mutation of gene segments. Innate immunity comprises of epithelial barrier, NK cell, phagocytes and complement system. Adaptive immune response is further characterized into cell-mediated immunity mediated by T lymphocytes and humoral immunity mediated by antibody producing B cells, which function jointly to eliminate different types of microbes.

1.1 The role of CD4+ T cells in antigen recognition and immune surveillance

Specific and precise recognition of antigen by lymphocytes, initiates adaptive immune response. Antigen recognition in adaptive immunity is mediated by antibodies (Abs), T cell antigen receptors (TCR), Major histocompatibility complex (MHC). CD4+ T cells play a central role as they control and regulate the adaptive immune response.

1.1.1 Activation of CD4+ T cells

Effective and potent immune response, require proper activation of CD4+ T cells. After the thymic development of T cells, they enter into blood stream and migrate towards peripheral lymphoid organs, and are termed as naive T cells. For adaptive immune response these naive T cells, get induced to proliferate and differentiate, to become armed effector cells. The first contact of antigen presented by professional APC to T cells results in primary immune response, and generates immunological memory. In case of CD8+ T cell activation, the antigen gets presented by MHC class I molecules at APC surface, providing license to CD8+ T cells for killing infected target cells. However CD4+ T cells get activated when APC present antigenic peptide in association with MHC II molecule. This interaction of their T-cell receptors with antigenic peptide/MHC II complex, on the surface of APC with simultaneous delivery of co-stimulatory signal by specialized APC, leads to CD4+ T cell activation. The density of MHC-II/antigen complex formed on the APC surface, is directly associated with the peptide dose (Kim, et al., 1996; Valitutti, et al., 1995; Vidal and Allen, 1996). The strength of interaction between APC and T cell, will directly affect effector functions of T cells, like proliferation and cytokine production (Valitutti, et al., 1995), and deletion of activated T cells might happen for very high antigen concentration (Critchfield, et al., 1994; Rotzschke, et al., 1997). CD4+ T cell activation is a critical step in adaptive immunity, directing the immune response against the invading pathogens and tumorogenic cells, in order to maintain immunological homeostasis (Wieder, et al., 2008). Upon activation naive CD4+ T cell can be polarized to either of Th subsets, Th1 or Th2 phenotype, depending on the cytokine produced during

their stimulation. PreciseTh1 stimulation produces IFN-γ leading to cell mediated immunity while Th2 produces IL-4, IL-13, IL-25 providing humoral immunity (Fort, et al., 2001; Mosmann and Coffman, 1989). Recently a third Th subset has been proposed called Th17, producing IL-17 (Langrish, et al., 2005), having characteristic effector functions.

Another category of CD4+ T cells which suppress the response of effector T cells are called as regulatory T cells (Tregs) (Sakaguchi, et al., 1995). Natural Tregs have a specific molecular marker Foxp3. Foxp3 Tregs are actively involved in negative control of a number of physiological and pathological immune responses. CD4+ and CD8+ T cell proliferation and production of effector cytokine regardless of TCR specificity can be suppressed by Tregs. (Piccirillo and Shevach, 2001; Thornton and Shevach, 2000). Tregs can be utilized for prevention or treatment of autoimmune diseases and also to induce immunological tolerance to non self antigens (in cases of transplantation tolerance), reducing abnormal immune response (allergies), boost host defense (in tumor immunity) (Sakaguchi, 2004). Tregs have multiple mechanisms to suppress proliferating immune response (Bluestone and Hebrok, 2008; Bluestone, et al., 2008; Bluestone, et al., 2008). Immunological balance is required between effectors and regulators for normal functioning of immune system.

1.1.2 Signalling in T cell activation

Activation of CD4+ T cells involves number of key players. Binding of CD4 to invariant sites on MHC II molecule outside the binding groove, enhances T-cell sensitivity to antigen. Integrins, selectins, immunoglobulin super family and some mucin like molecules facilitate the interaction of T cells with other cells. Key role in maintaining immunological homeostasis is played by cytokines secreted by the activated helper T cells. Intercellular adhesion molecules (ICAM) namely ICAM-1, ICAM-2 and ICAM-3. Binding of professional APC and dendritic cells to naive T cells, involves interaction between LFA-1 (lymphocyte function associated antigen-1), CD2 and ICAM-3 present on T cells and LFA-1, LFA-3, ICAM-1 and ICAM-2 on APC. The activity of peripheral mature T cells can be tailored by positive and negative regulatory receptors (Sharpe and Freeman, 2002). Positive modification of T cell activation involves co-stimulatory signal, which is provided by the same APC, which present the antigen to TCR via MHC. B7.1 (CD80) and B7.2 (CD86) called as B7 molecules are the best characterized co-stimulatory molecules, which interact to CD28 present on naïve T cells, providing positive signal, thereby lowering the threshold for TCR signalling (Freeman, et al., 1993; Freeman, et al., 1993). This promotes expansion and differentiation of T cells with enhanced T cell cytokine response (Sperling, et al., 1996; Thompson, et al., 1989). Professional APC like dendritic cells are the very potent activators of naive T cells, as they have high levels of MHC I, MHC II, co stimulatory molecule B-7, as well as adhesion molecules ICAM-1, ICAM-2 LFA-1 and LFA-3. Differentiation and proliferation of armed effector T cells, is driven by a key cytokine or protein growth factor interleukin-2 (IL-2), produced by activated T cells, in presence of co-stimulatory signal. This growth factor activates T cell proliferation to form progenies expressing identical antigen receptor. Presentation of antigen to T cell in absence of co-stimulatory molecules prevents T cell activation and also makes T cells anergic. Another receptor present on activated T cells called CTLA4 counteracts the stimulatory effect of CD28 ligation providing negative signal to T cells, reducing proliferative response and interleukin-2 (IL-2) production (Brunet, et al., 1987; Krummel and Allison, 1995; Linsley, et al., 1991). Thus to sum up a perfect balance between CD28 and CTLA-4 linked signals are crucial to T cell activation and tolerance.

1.2 Major histocompatibility complex (MHC)

MHC molecules are peptide receptors involved in transferring the protein information from inside of the cell to the surface. Antigen presented in context to MHC molecules decides, which subset of T cells have to be activated. When antigen is presented in context to MHC I proteins, CD8+ T cells are activated while CD4+ T helper cells are activated when antigens are presented in context of MHC II proteins. MHC I molecules are expressed in most of the soma cells, however expression of MHC II proteins is restricted to thymic epithelial cells and professional APC (mainly dendritic cells, B cells and macrophages).

1.2.1 Genome organisation of MHC proteins

Human MHC (HLA) genes, are located on short arm of chromosome 6 (Ljunggren and Karre, 1985), with gene mapping to a 3.8kb DNA stretch on p21.3 band, however mice MHC (H2) genes are located on chromosome 17. The MHC (HLA) class I region is known to span approximately 1.8kb with HLA(A) being most telomeric, and located on short arm of chromosome (Colonna, 1996; Karre, 1995). The HLA class II region consists of three major sub regions HLA-DP, HLA-DQ, HLA-DR, arranged in direction from centromere to the telomere spanning at least 800kb (Karre, 1993; Long, et al., 1996). The HLA-DR sub region length varies between 60 to 260kb, having one DRA and many DRB genes. DRB genes are extremely polymorphic while DRA gene is non polymorphic except one exception of leucine substitution by valine at position 217 (Cossins, et al., 1993; Robbins, et al., 1989).
The HLA-DQ sub region consists of two pairs of DQA and DQB genes, with both A and B genes being polymorphic (Oldstone, et al., 1988). HLA-DP genes are present in two subsets within the sub region. Only DPA1 and DPB1 genes are expressed as product, while DPA2 and DPB2 genes are nonfunctional pseudogenes, consisting of deleterious mutations in coding sequence (Rask, et al., 1991).

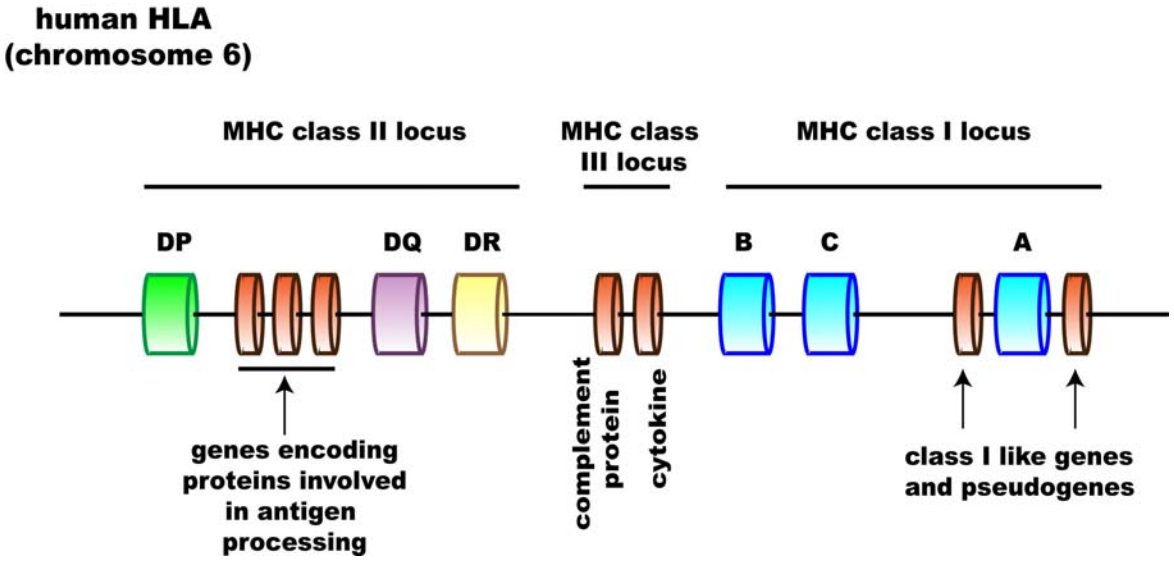

Figure 1: Genome organization. Complete picture of the genome organization of MHC locus comprising of MHC I, II and III locus, present in chromosome 6.

1.2.2 MHC class I structure and antigen processing

MHC class I molecules consist of 46kDa polymorphic type I integral membrane glycoprotein heavy chain, non-covalently linked with a 12kDa soluble subunit β2-microglobulin (β2m) (Bjorkman and Parham, 1990). Two distinct extracellular structural units are present in heavy chain, first membrane distal α1 and α2 domain, which pair to generate a cleft that forms the peptide binding region, for antigen binding and membrane proximal α3 domain forms a loop that binds to CD8 (Evans, et al., 1994; Moore, et al., 1982). β2m is a single, folded and compact immunoglobulin like domain without any membrane anchor, and is found either linked to heavy chain or stays free as soluble fraction in tissue fluid and plasma. The α1 and β2 domain form single peptide binding groove supported by a floor of β-pleated sheets having eight strands, and enclosed by two α helices (α1 and α2). β2m makes contact both with conserved immunoglobulin like α3 domain and also with β sheet floor of the α1 and α2 antigen binding groove. (Garrett, et al., 1989). Hence fully assembled MHC molecule has α-chain, β2-microglobulin and a bound antigenic peptide, and this heterotrimer is expressed at cell surface in form of functional MHC, presenting antigen to CD8+ T cells.
Antigen processing of MHC class I is different than MHC class II molecule. MHC I binds peptides during early assembly in the endoplasmic reticulum (Cresswell, 2005; Rock, et al., 2004). Proteosomes generate short peptides having carboxy terminus residues from the proteolytic degradation of endogenous proteins, (Rock, et al., 2004; Shastri, et al., 2002). The peptides thereby get transported via heterodimeric transporter associated with antigen processing (TAP) into the endoplasmic reticulum lumen. However final amino terminal trimming of peptides is done by endoplasmic reticulum amino peptidases ERAAP or ERAP 1 (Falk and Rotzschke, 2002; Serwold, et al., 2002; York, et al., 2002) serving as a 'molecular ruler' (Chang, et al., 2005). Components involved in MHC class I loading complex are tapasin (peptide editor), calreticulin, TAP and thiol oxidoreductase ERp57 and newest component protein disulfide isomerase (PDI) (Raghavan, et al., 2008; Wearsch

and Cresswell, 2008). Once stable MHC class I/peptide complex is formed, it is transported through golgi cisternae, ultimately to cell surface via constitutive secretory pathway, for antigen presentation to CD8+ T cells (Liu and Gao, 2008).

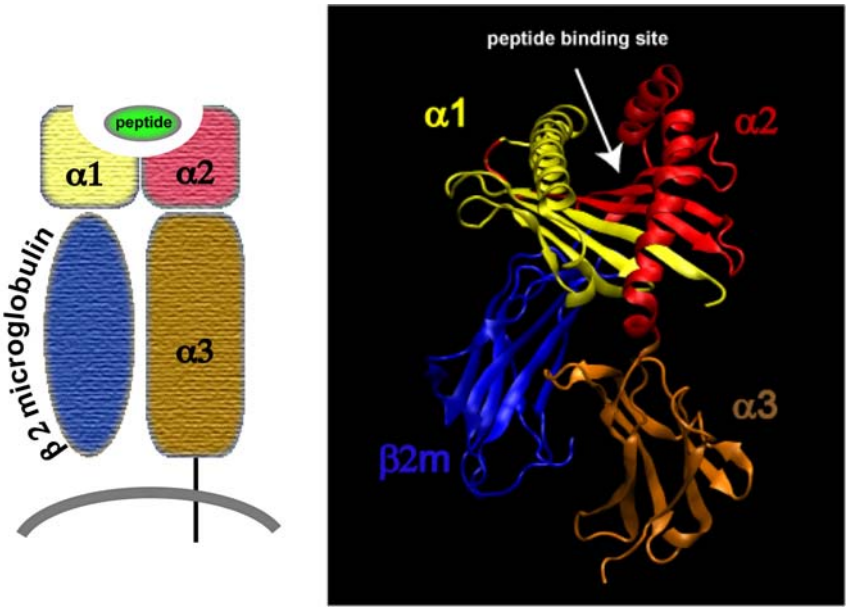

Figure 2: Structure of extracellular domains of MHC class I protein, without peptide. Complete view taken from crystal structure of MHC class I H-2Kb molecule complexed with pKB1 peptide, modified accordingly (figure right panel) and cartoon representation (figure left panel). Functional empty MHC class I molecule is formed by pairing of α-chain and β2-microglobulin. α-chain comprises of α1 (yellow), α2 (red) and α3 (brown) domain and interacts with β2-microglobulin (blue). α1 and α2 domains interact to form the antigen binding site (pdb: 1kj3).

1.2.3 MHC class II structure

MHC class II proteins are type I heterodimeric integral membrane proteins (Kaufman, et al., 1984). Similar to MHC I, organization of intron and exon coding MHC II α and β chains, well corresponds to protein functional domains. The coding information for bulk protein, contained within exon-2, with extensive polymorphism within species (Benoist, et al., 1983; Choi, et al., 1983). Each heterodimer is composed of covalently linked α-chain of 32 to 34kD, and β-chain of 29 to 32kD. The peptide binding cleft in MHC II proteins is formed by the interaction of amino terminal of α 1 and β 1 domains. α1 contributes four strands of the floor and one helix, and remaining four strands and second helix is contributed by β1. α1 and β1 segments are polymorphic. In humans β-chain is highly polymorphic. α2 and β2 domains are folded in form of immunoglobulin like domains, and are non-polymorphic. A loop formed in β2 domain is the binding area for CD4. α2 and β2 domains carboxy terminal end continues into short connecting area, subsequently followed by a stretch of transmembrane hydrophobic residues. End part of both transmembrane α and β chain, are basic amino acid residues and hydrophilic cytoplasmic tail. Completely

assembled MHC molecules have α-chain, β-chain and bound antigenic peptide (Germain and Margulies, 1993; Kaufman, et al., 1984). This heterotrimer is expressed on the cell surface in form of functional MHC II receptor, presenting antigen for CD4+ T cell surveillance.

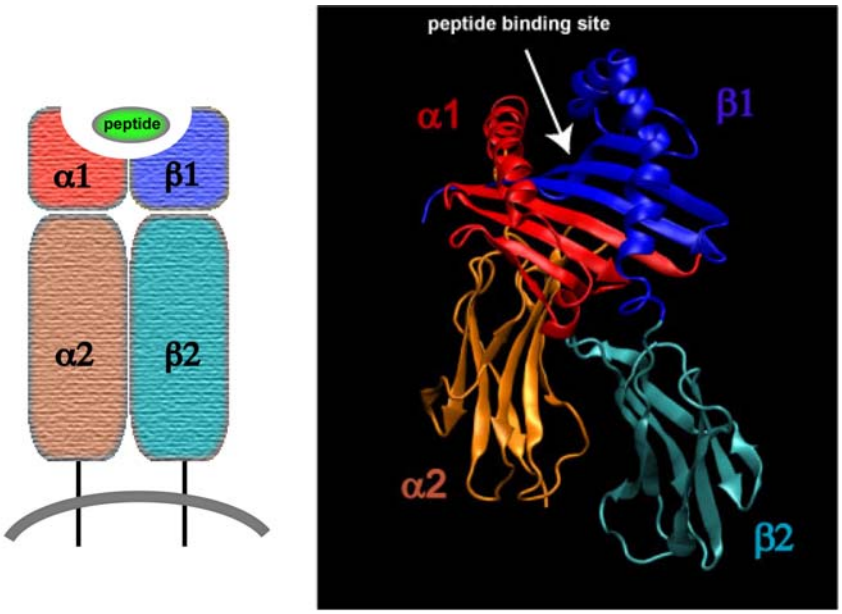

Figure 3: Structure of extracellular domains of MHC class II protein, without peptide. Complete view taken from crystal structure HLA-DR1/HA306-318, modified accordingly (figure right panel), cartoon representation (figure left panel). MHC class II molecule is formed by two chains α and β, and both chains participate to formcomplete functional MHC class II molecule. α-chain comprise of α1 (red) and α2 (orange) domain and β-chain is comprised of β1 (blue) and β2 (cyan) domain. α1 and β1 domains form the peptide binding site (pdb:1dlh).

1.2.4 MHC class II: synthesis and antigen processing pathways

1.2.4.1 Endosomal pathway

The α and β subunits of MHC II molecules get associated within 2 minutes of their synthesis, (Kvist, et al., 1982) and utilize conserved characters of transmembrane regions of the polypeptide chains (Cosson and Bonifacino, 1992). In physiological conditions α and β-chains, of MHC molecule co-assemble, in the presence of invariant chain (Ii) (Jones, et al., 1979). Ii chain is type II non-polymorphic membrane glycoprotein, having NH_2 terminus buried in cytoplasm and COOH terminus in the lumen of ER (Singer, et al., 1984; Strubin, et al., 1984). In the ER, Ii forms noncovalently interacting trimers, so that MHC class II αβ dimer get associated with the trimer. This unstructured segment part of the Ii, fits in to the peptide binding groove of MHC II, and facilitates smooth transport of αβ Ii to endosomes, prevents any non specific interaction of misfolded polypetides with MHC binding groove. Most importantly it prevents any ER ligand to bind MHC II protein (as in principle ER ligand is supposed to associate to MHC I molecules) (Layet and Germain,

1991; Lotteau, et al., 1990; Teyton, et al., 1990; Viville, et al., 1993). The αβ multimeric assembly, comes out of ER and then get transported via golgi stacks (Machamer and Cresswell, 1982; Sung and Jones, 1981). αβ Ii complex on reaching to the trans-golgi network get deviated from the normal exocytic pathway, and utilize endocytic pathway to reach cell membrane (Neefjes, et al., 1990). The targeting signal in the cytoplasmic domain of the Ii, guide their way into the endo-lysosomal pathway (Roche and Cresswell, 1990). αβ-Ii complex is concentrated mostly in the multivesicular and multilamellar, late endosomal compartment which is also referred to as 'MIIC' (Rocha and Neefjes, 2008). By the action of proteolytic enzymes mainly cathepsin S, L F and aspartil and cysteinil proteases, Ii is cleaved (Bryant, et al., 2002). After Ii cleavage MHC α β heterodimer is released from Ii cytoplasmic tail endosomal retention signal. Cleavage of Ii, leaves behind a short segment of Ii 81-104, class II associated invariant chain peptide (CLIP), which remain attached to the antigen binding groove of the MHC II molecules (Riberdy, et al., 1994; Riberdy, et al., 1992). The antigenic peptides generated inside the endosomes, due to endocytosis of proteins are loaded on to the MHC II ligand binding groove by the exchange of CLIP (Kropshofer, et al., 1995). Exchange of CLIP with antigenic peptides occurs at acidic medium, pH value around 5.0 due to favourable structural transitions in the MHC molecule for peptide loading (Boniface, et al., 1996). Reduced pH favours the activity of natural catalyst for ligand loading and exchange known as 'HLA-DM' (or H-2M for mouse), serving as CLIP fragment remover, and facilitating binding of kinetically stable antigen repertoire, generated in endosomes. (Fling, et al., 1994; Kropshofer, et al., 1996; Morris, et al., 1994; Sloan, et al., 1995). After loading with antigenic peptides MHC class II molecules are transported to cell surface for antigen presentation (Berger and Roche, 2009). MHC Class II proteins can be found expressed on B cells (Guagliardi, et al., 1990; Peters, et al., 1991), activated macrophages (Harding, et al., 1990), golgi stacks, trans golgi network, plasma membrane, and in intracellular vesiscles or early endosomes (Guagliardi, et al., 1990)

1.2.4.2 Cell surface loading

By default peptides are loaded on MHC class II proteins in endosomal retention pathway but alternate pathway for trafficking and loading of MHC II proteins have also been described. This alternate pathway involves recycling of MHC II proteins from cell surface (Walseng, et al., 2008). Experiments done with fixed cells or MHC expressing cells lacking important components of the processing pathway indicate that MHC loading can also take place directly on the cell surface. This is valid not only for optimally sized peptides but also for larger polypeptide chains or even full-length proteins such as S-methylmyoglobin, myoglobin, RNAse or myelin basic protein (Marin-Esteban, et al., 2003; Pinet, et al., 1995; Vergelli, et al., 1997) partially denatured MBP or unfolded proteins like fibrinogen (Allen and Unanue, 1984; Pinet, et al., 1995). To form stable cell surface MHC/Peptide complex, the loaded protein has to be further processed. The processing of this endocytosed antigen takes place in the endosomes (Allen and Unanue, 1984; Pinet, et al., 1995).. In particular immature dendritic cells (DC) could utilize this pathway. As these immature DC contain a large fraction of 'empty' class II MHC molecules on the cell surface, which may allow the direct capturing of antigens from the extracellular space. Thus these 'empty' MHC class II proteins can act as antigen receptors, by collecting extracellular peptide antigens, which escape pinocytosis. Empty, 'peptide receptive' MHC class II proteins, at cell surface can bind and present low affine ligand, that are unable to compete for binding inside endosomes (Santambrogio, et al., 1999;

Santambrogio, et al., 1999). This function can be important in maintaining peripheral T cell tolerance and also in thymic T cell selection than in presentation of foreign antigens at lymph nodes. During maturation DC undergo several important changes including redistribution of MHC class II molecules, upregulation costimulatory signals, lysosomal acidification (Trombetta, et al., 2003), enhanced antigen uptake, as a result of TLR signaling (West, et al., 2004). Fraction of cell surface expressed MHC class II molecules, are also found to stay in lipid raft like membrane microdomains (Anderson, et al., 2000; Hiltbold, et al., 2003) with subgroup of 'tetraspan' microdomains, selectively recruiting MHC class II subset (Kropshofer, et al., 2002). Composition of their peptide repertoire is different to that presented by MHC molecules that are present outside lipid rafts (Hiltbold, et al., 2003; Kropshofer, et al., 2002). It is observed that α β Ii-complex first reaches cell surface and then it comes back to endosomes where CLIP is exchanged for antigenic peptides (Benaroch, et al., 1995) and AP2 has been shown as key element to deliver cell surface αβ Ii to endosomes as its AP2 blocking increased αβ Ii (Dugast, et al., 2005).

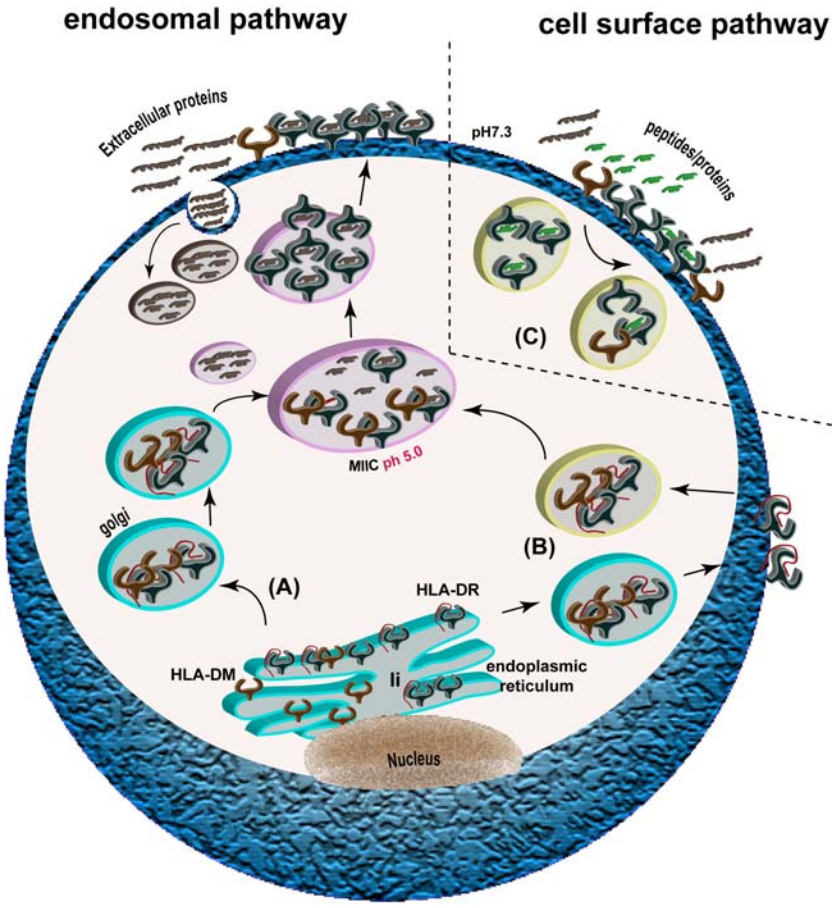

Figure 4: MHC class II antigen processing pathways. Antigen loading on MHC class II molecules can take place by the default endosomal pathway (A) or by direct cell surface loading pathway (C) or pathway that takes MHCII-Ii to cell surface and then back to endosomes or recycling pathway (B). (A) In the endoplasmic reticulum (ER) the MHC class II molecules get assembled with Ii, and then get transported to late endosomes or MHC class II compartment (MIIC). Due to acidic condition and proteases the Ii gets cleaved into CLIP. Natural catalyst HLA-DM, loads antigenic peptide by exchanging with

CLIP. Finally MHC/antigen complex is then transported to cell surface. (B) MHC II-Ii complex together with HLA-DM might first go to cell surface. Further the complex gets internalized and finally gets exchanged with antigenic peptide in endosomes in HLA-DM dependent manner. (C) On cell surface empty MHC class II molecules may directly bind antigenic peptides, which can be further processed in the endosomes. HLA-DM present on the cell surface gets co-internalized and might stabilize the complex intracellularly. Cell surface MHC molecules might also form complex with some extracellular antigens directly on cell surface, without any further processing.

1.2.5 Peptide binding and stabilization

Peptide antigen/MHC complexes are a central element of the cellular immune system. The binding groove on MHC-II proteins can bind minimum 9 amino acid long core of a short peptide or a protein. The binding cleft of MHC class II proteins is open from both the ends, therefore peptides can extend at both N and C-termini, without restriction in maximum length (Chicz, et al., 1992; Rudensky, et al., 1992; Stern, et al., 1994). The conserved MHC residues interact with the peptide backbone, and apparently stabilize the peptide in polyproline type II conformation, throughout the length of peptide binding groove (Stern, et al., 1994). The polymorphism in MHC class II binding site plays a very important role in antigen binding, and allele succeptibility, as it dictates specific preferences and restrictions of individual pockets. Peptide binding site of MHC class II molecules have four predominant pockets namely P1, P4, P6 and P9. Residues P3 and P7 are held by shallow pockets on the surface of the binding site and mutually with P2, P5 and P8 which project out of the binding site, these residues are involved in forming contact with TCR (Stern, et al., 1994). Peptide is stabilized in the binding groove of MHC molecules mainly by the main chain H-bond interaction and anchor side chain interactions. Hydrogen bonds present in the peptide N-terminal part, appears to be more critical than at C-terminal (McFarland and Beeson, 2002). These H-bonds provides stability to peptide in MHC binding groove (O'Sullivan, et al., 1990; Sato, et al., 2000). H bond interactions are also targeted by HLA-DM to dissociate prebound ligand from the complex (Narayan, et al., 2007). Additionally, at least for HLA-DR1 overall peptide binding is dominated by pocket-1 (O'Sullivan, et al., 1990; Sato, et al., 2000). Therefore P1 pocket plays critical role in peptide stability and conformational transition.

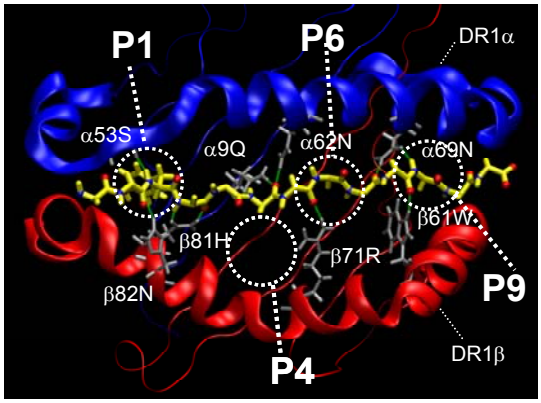

Figure 5: Structure of the binding site of MHC class II/peptide complex showing MHC pockets and H-bond networks. Top view of HLA-DR1/HA 306-318 complex (pdb :1dlh). The depictions are as follows α1: blue ribbon, β1: red ribbon. Position of pockets have been depicted in white dotted circles, showing pocket P1, P4, P6 and P9 respectively. Peptide backbone HA306-318 is shown in yellow. MHC residues forming H-bonds with the peptide backbone are labelled in grey. (Gupta, et al., 2008)

1.3 Conformational transitions in MHC class II proteins

The MHC class II conformations are dynamic, and are influenced by number of factors like important components involved in antigen processing and presentation (Vogt, et al., 1997; Weber, et al., 1996), certain alcohols (Marin-Esteban, et al., 2004), chemical compounds (Hopner, et al., 2006), and pH (Boniface, et al., 1996) or unstable kinetic intermediates emerging before the formation of stable MHC/peptide complex (Sadegh-Nasseri, et al., 1994). These conformational transitions, in MHC proteins, provide specific function. Antigen loading in the endosomes is regulated by acidic pH, which results in structural changes in MHC proteins (Boniface, et al., 1996; Denzin and Cresswell, 1995; Sherman, et al., 1995), causing enhanced antigen loading (Boniface, et al., 1996). Binding of peptide ligand to MHC proteins also cause conformational shift (Bluestone, et al., 1992; Chervonsky, et al., 1998; Reich, et al., 1997; Solheim, et al., 1995), evident by spectroscopic and hydrodyanamic studies (Joshi, et al., 2000; Zarutskie, et al., 1999). Dipeptide binding (Sato, et al., 2000) or filling pocket 1 (Natarajan, et al., 1999), also leads to conformational change. Size and sequence of peptide ligand also affects the MHC class II/ligand complex conformation (Sadegh-Nasseri and Germain, 1991; Sadegh-Nasseri and McConnell, 1989). Binding of extended peptides to HLA-DR too forms different conformational isoforms, floppy and compact form, differing in hydrodynamic radii (Rotzschke, et al., 1999). This floppy and compact structure, between MHC class II/peptide can be distuingshed (Sadegh-Nasseri and Germain, 1991; Sadegh-Nasseri and McConnell, 1989). Different conformational variants with floppy and compact form, of same MHC/peptide complex can be recognized as short lived or more stable complex (Sadegh-Nasseri, et al., 1994). T cell clones do selectively recognize these conformers (Rabinowitz, et al., 1997). Positive selection in thymus can also be affected by 'floppy' MHC/peptide complex (Viret, et al., 2003). Encephalitogenic T cells show dissimilar recognition to murine MHC class II molecules, loaded by mutated peptide antigens in

experimental autoimmune encephalomyelitis (EAE) model (Huang, et al., 2003). Differential recognition to different set of T cell clones is observed in the case of isoforms of antigen/MHC complex, containing same peptide ligand (Pu, et al., 2002; Pu, et al., 2004). Thus conformation variants in MHC class II proteins play a very critical physiological role, in antigen recognition and presentation.

1.3.1 Receptive and non-receptive MHC conformation states

Two critical MHC conformers exist, a long lived inactive 'non receptive' state and short lived active form termed as *'receptive'* state (Natarajan, et al., 1999; Rabinowitz, et al., 1998; Zarutskie, et al., 1999). Empty MHC II molecule rapidly adopt the 'non receptive state', therefore loading of antigen directly on MHC class II proteins is very slow process (Stern, et al., 1994), due to first conversion of 'non receptive state*'* to active 'receptive state*'*. The short lived peptide receptive MHC conformation is formed immediately after the loss of bound ligand, characterized by rapid binding of added ligand, however *'*peptide receptive state*'* converts to 'non receptive state' in the absence of any ligand (Rabinowitz, et al., 1998). 'Receptive state' is characterized by fast on and off rate of ligand binding, (Natarajan, et al., 1999; Rabinowitz, et al., 1998). Binding of peptide to 'non receptive' MHC molecule is much slower (Joshi, et al., 2000). Conversion of receptive state from non receptive is rather very slow, however HLA-DM a natural chaperone (Sadegh-Nasseri, et al., 2008) and also simple chemical compounds stabilize the receptive state in MHC class II proteins, resulting in elevated ligand exchange (Hopner, et al., 2006; Marin-Esteban, et al., 2004) . Their is equilibrium shift of closed conformation or 'non receptive' to open conformation or 'receptive' under acidic conditions (Jensen, 1990; Sadegh-Nasseri and Germain, 1991) and these conformational variants are supposed to play very critical role in interaction with HLA-DM and also peptide loading (Denzin and Cresswell, 1995; Kropshofer, et al., 1996). 'Receptive' and 'non-receptive' form are conformationally different, and even conformational sensitive antibodies are able to probe the structural changes by showing reduced reactivity to peptide/MHC complex (Carven, et al., 2004; Hansen, et al., 2005; Santambrogio, et al., 1999), but none of the antibodies have been reported to probe the 'receptive conformer' of MHC molecule. Lack of crystal structure of receptive MHC molecule, also restricts our structural information regarding receptive state. However P1 pocket is supposed to play an important role in stabilizing receptive state (Hopner, et al., 2006; Rupp, unpublished), as it plays pioneer role in peptide binding. Its occupation by side chain of amino acid is sufficient to diminish conformational sensitive antibody interaction (Chou and Sadegh-Nasseri, 2000; Sato, et al., 2000). Permanent filling of pocket 1* makes MHC receptive (Natarajan, et al., 1999), with HLA-DM showing no effect on '*receptive'* MHC molecule (Narayan, et al., 2007), pointing towards crucial role played by Pocket 1 in the conversion of 'non receptive' to 'receptive' or vice versa. Loss of ligand at APC surface converts MHC into 'non receptive' state and this works as safeguard mechanisms, in preventing accidental antigen presentation from outside environment, due to the fact that MHC molecules have to reflect the inside content of cell, rather than outside environment (Stern, et al., 2006). Recently a model of peptide free conformation is also suggested (Painter, et al., 2008; Yaneva, et al., 2009). In summary 'receptive' *a*nd 'non receptive' MHC conformers play decisive role in antigen binding and presentation.

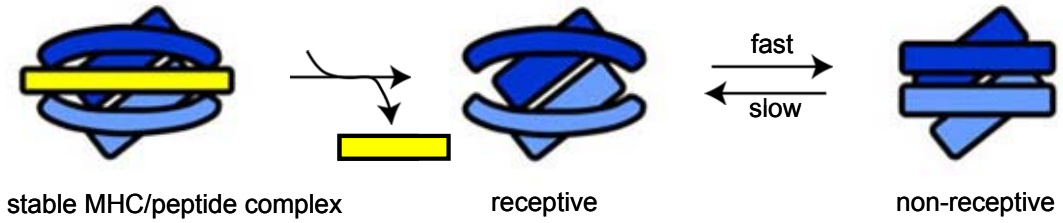

Figure 6: Receptive and non receptive conformational state of MHC class II proteins. Figure depicts receptive and non receptive MHC conformers. MHC/ligand complex converts quickly into short lived "receptive state" after loss of ligand, and then quickly converts into "non receptive" or "closed conformer". Modified from (Grotenbreg, et al., 2007)

1.4 Mediators of the MHC class II conformation transition

1.4.1 HLA-DM

The genes encoding HLA-DM- DMA and DMB have been mapped in the MHC class II region, but differ from MHC II genes in various ways (Cho, et al., 1991; Kelly, et al., 1991). The crystal structure of HLA-DM (Mosyak, et al., 1998) shows the absence of pockets, for binding of side chains of ligand, and also absence of conserved residues (as present in HLA-DR) (Stern, et al., 1994) that make contact with the ligand. Targeted and random mutagenesis (Pashine, et al., 2003) and *in silico* molecular docking (Davies, et al., 2008) experiments gave extensive information on the topology of DM/DR interaction. Co-crystals of HLA-DM and MHC II protein are not present uptil now but it would be very valuable in throwing light on atomic resolution of DM catalyzed peptide release. The peptide exchange mediated by HLA-DM, follows Michaelis-Menten kinetics (enzyme catalysis), supporting the idea that DM too acts like catalyst (Vogt, et al., 1996). HLA-DM significantly increases CLIP release, thereby enhancing binding of substitute antigenic ligand to MHC class II proteins (Denzin and Cresswell, 1995; Sherman, et al., 1995; Sloan, et al., 1995). DM enhances peptide release by targeting conserved hydrogen bonds specially β 81-His residue (Narayan, et al., 2007). Peptide dissociation can also be promoted by disruption of single hydrogen bond, (McFarland, et al., 2001) but there exists co-operativity in hydrogen bond network (McFarland, et al., 1999). HLA-DM might also remove conformational isomers distinguished by T cells (Lovitch, et al., 2007; Lovitch, et al., 2006), and this edited conformer can be presented to T cells in vivo during inflammatory environment (Lovitch, et al., 2007). Experiments with alloreactive T cell clone (Katz, et al., 1996) and also with peptide mapping studies show that DM can edit the peptide repertoire qualitatively (Kropshofer, et al., 1996). Certain chemical compounds (Hopner, et al., 2006), alcohols (Marin-Esteban, et al., 2004) and noble metals (De Wall, et al., 2006) can also exchange ligand in DM independent fashion. The activity of HLA-DM has been recently questioned (Grotenbreg, et al., 2007), that DM forms complex with MHC II proteins contributing directly to peptide association. DM prevents the loss of ligand binding sites by stabilizing empty MHC proteins. HLA-DM stabilizes the *'receptive state'* of MHC class II proteins and thus catalyzes the peptide loading (Denzin, et al., 1996; Rabinowitz, et al., 1998). DM mediated MHC II stabilization also prevents loss of ligand

binding sites (Kropshofer, et al., 1997). Alternately DM can also work by hit and run mechanism, by releasing the bound ligand and subsequently selecting peptide that can form most stable complex (Narayan, et al., 2007). Predominantly DM is functionally active at endosomal acidic pH but studies claim the presence of DM (about 10% of total DM) on immature dendritic cell surface and also on B cell surface (Arndt, et al., 2000; Min, et al., 2000; Santambrogio, et al., 1999). Surface expressed DM accelerates the loading of exogenously added high affine ligands and reduces binding of low affine ligands (Arndt, et al., 2000; Pathak, et al., 2001). Suprisingly DM also counteracts the T cell presentation of myelin basic protein (autoantigen in multiple sclerosis) (Arndt, et al., 2000) that gets loaded on to the cell surface MHC class II proteins and in recycling compartments (Vergelli, et al., 1997). Activity of HLA-DM can be modulated by DO (HLA-DO or mouse homologue H-2DO), expressed in B cells and APC of thymic medulla (Alfonso, et al., 2003; Chen and Jensen, 2008).

1.4.2 ' MHC loading enhancer' (MLE) compounds

Studies carried out in the host lab have already shown that simple alcohols which are capable of disrupting hydrogen bond can efficiently catalyze both antigen loading as well as complex dissociation on HLA-DR1 molecules at neutral pH (Falk and Rotzschke, 2002). Subsequent studies with improved simple chemical compounds like parachlorophenol (pCP) showed that not only short peptides but larger polypeptides can be transferred on cell surface MHC molecules. It could be demonstrated that MLE directly affect the functional state of MHC by inducing peptide *'receptive state'* (Marin-Esteban, et al., 2004). High throughput screen (HTS) identified chemical compounds that can enhance antigen loading on HLA-DR protein by targeting polymorphic pocket 1. Remarkably, activity of the most potent compounds (like 1-Adamantane ethanol) correlated with a dimorphic residue on HLA-DR β-chain. Most of the compounds exhibited a strict allele-specificity. The activity of these compounds directly correlated to the β-86 residue located at the depth of P1 pocket. β-86 residue restricts the depth of pocket(Ong, et al., 1991). With these compounds activity was observed only on variants expressing glycine at β-86 position (β86G) with deep pocket but not on HLA-DR molecules expressing valine at β-86 position (β86V), with shallow pocket. Enhanced antigen loading could be directly translated into amplification of CD4+ T cell immune response in allele selective way (Hopner, et al., 2006).

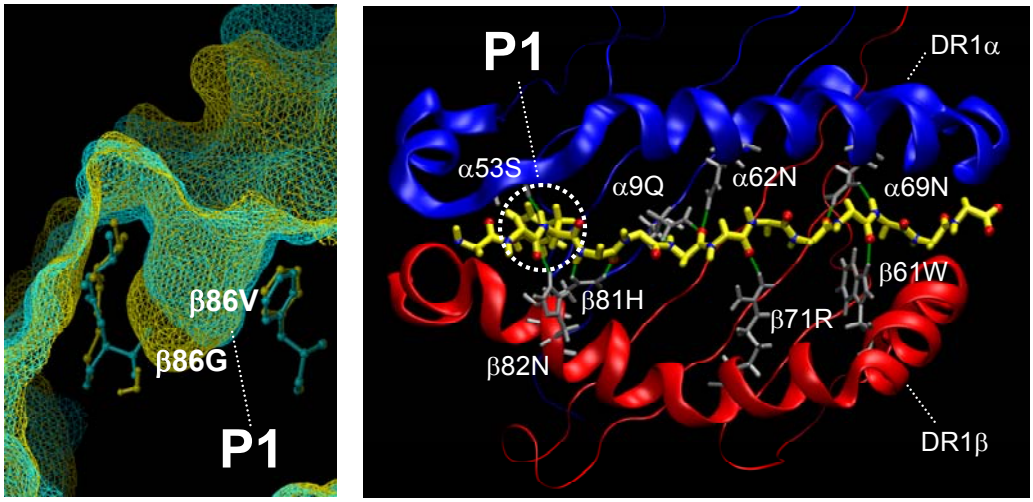

Figure 7: Representation of dimorphic residue β86 at base of pocket 1. Left panel shows the overlay of β86G residue (shown by mustard colour) β86V (green colour). β86 residue with glycine has deep pocket 1 while valine residue makes P1 pocket is shallow (Hopner, et al., 2006). Right panel shows crystal structure HLA-DR1/HA306-318 complex showing pocket 1 (pdb code:1-dlh) (Gupta, et al., 2008; Stern, et al., 1994).

MLE compounds 'open up' the binding site of human class II MHC molecules by specific interaction with pocket 1. By this mechanism these 'MHC loading enhancers (MLE) can recover inactive MHC molecules by providing additional free binding site by triggering the release of low affine endogenous ligands. In a mechanistic model the phenomenon could be explained by assuming that the 'receptive' form is correlated with an open pocket 1, while the 'non-receptive' state is linked to collapsed pocket 1. On the basis of this assumption MLE compounds, should transiently occupy the P1 pocket, this would open P1 pocket and also prevent its collapse. This would stabilize the 'receptive state', resulting into fast peptide loading rates as a result of an amplified pool of peptide accessible 'receptive' MHC molecules (Hopner, et al., 2006).

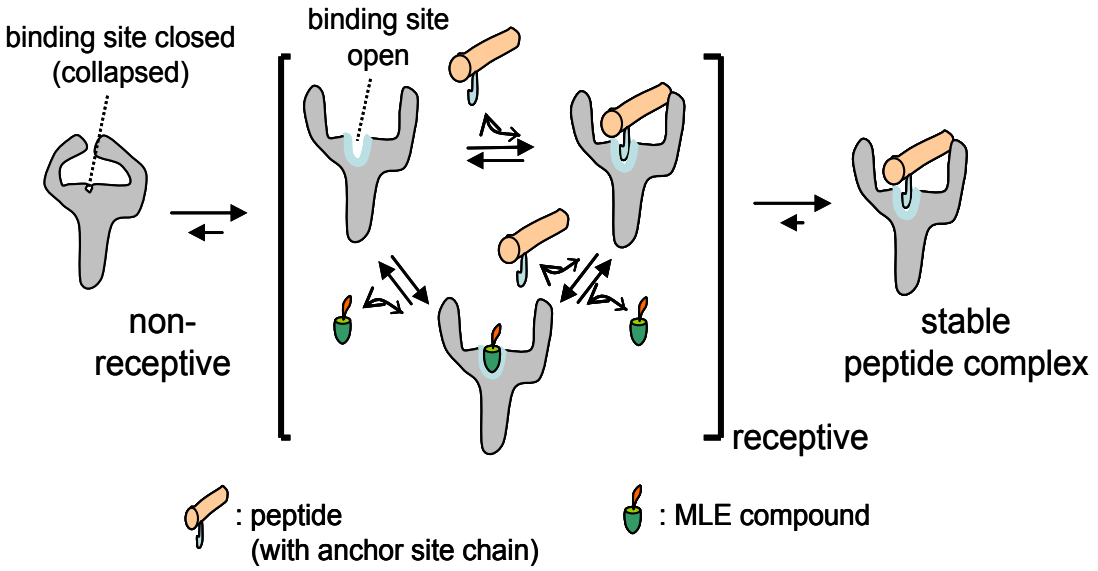

Figure 8: Mechanistic model of MLE mediated antigen loading. Loading of peptide on MHC class II molecules is a multistep process, involving conversion of the 'non-receptive' state into the short lived 'receptive' state (Joshi, et al., 2000; Sadegh-Nasseri, et al., 1994). According to this model the peptide receptive conformation, required for antigen loading is correlated with open P1 pocket, while non receptive with collapsed P1 pocket. A filled pocket 1, should therefore suggest stabilization of the receptive state. Transient occupation of pocket 1, by MLE compounds should therefore increase the number of peptide-accessible MHC molecules, by preventing the reconversion into inactive 'non-receptive' conformer (Hopner, et al., 2006).

As 'MHC loading enhancer' (MLE) they represent useful tools to enhance antigen loading in a variety of diagnostic and therapeutic settings. This might include the multimeric MHC molecule loading e.g. HLA-DR tetramer, (Altman, et al., 1996) or enhancing antigen loading on APC surface for *in vitro* T cell assays or immunotherapies. They might also be used as 'drug like' vaccine *'additive'* in various therapeutic settings.

1.5 Environmental factors and autoimmune disorders

Environmental triggers like viruses, bacteria and other infectious pathogen are thought to play important role in autoimmune disorder development (Ercolini and Miller, 2009). It is known that number of drugs can adversely affect and, may result in appearance of autoantibodies, and might also show up with range of autoimmune clinical syndromes. Some of the chemical factors like contaminated rape seed oil cause toxic oil disease (Vicario, et al., 1982) aromatic amines and hydrazines, containing drugs such as procainamide and hydralazines, causing lupus (Batchelor, 1980; Brand, et al., 1984; Mongey and Hess, 2007; Russell, et al., 1987; Speirs, et al., 1989). Hydrazine sulphate and tartrazine causing lupus like syndrome (Reidenberg, 1981; Reidenberg, 1983), high dose or prolonged treatment with paraphenylenediamine, a hair dye causing scleroderma-like lesions in animals (Geschickter, et al., 1958). Cases of SLE (Systemic lupus erythematosus) with high exposure to silica (Conrad, et al., 1996) is also observed.

Development of scleroderma, occupational acro-osteolysis with vinyl chloride (Dodson, et al., 1971). Good pasture's syndrome (autoimmune kidney disorder) related to polyhalogenated hydrocarbons, exposure commonly present in paints and varnishes (Beirne and Brennan, 1972). Pristane a compound of mineral oil can induce antibody production with lupus like syndrome, in some inbred mice strains (Satoh, et al., 1996). Strong association to HLA-DPB1*0201 allele for chronic beryllium disease (CBD), due to beryllium exposure (commonly present in ceramics,electronics) (Richeldi, et al., 1993). These chemicals can mediate the immune hazard by several mechanism to modify normal functioning of immune system. This might result due to polyclonal B cell activation which in turn produce autoantibodies in succeptible individuals or generating free radicals causing inflammation. They can also cause toxicity to immune cells. Epigenetic modification of T cell DNA, could also happen causing autoreactive T cells and autoimmunity. (Hess, 2002; Mongey and Hess, 2007).

1.6 MHC linkage to various diseases

Genetic and functional studies support the MHC II linkage to various diseases like celiac disease found to be linked with HLA-DQ2 (85%) and HLA-DQ8 (10-15%), (Sollid and Thorsby, 1993). Narcolepsy to HLA-DQ6 (Mignot, et al., 2001), Type 1 Diabetes to HLA-DQ8 in human and IA^{g7} in NOD mice (Hattori, et al., 1986; Nepom, 1993; Todd, et al., 1987), and some others are mentioned in the table below.

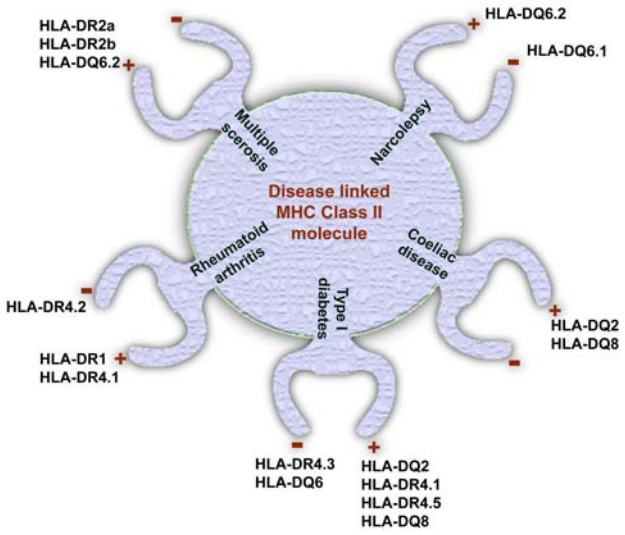

Figure 9: Disease linked MHC class II proteins. a) '+': suggests positive association meaning corresponding MHC alleles enhance the susceptibility to particular disease b) '-': suggests negative association, meaning corresponding MHC allele protects against disease.

HLA complex genes other than coding for antigen presenting HLA proteins, may also contribute to predisposition of disease (Horton, et al., 2004). Linkage disequilibrium, in

HLA complex creates major hurdle to uncover the importance of other HLA complex genes. Additional genes from extended MHC I region, may also predispose to celiac disease (Lie, et al., 1999) or type 1 Diabetes (Lie, et al., 1999). Several other studies also show the importance of extended HLA complex , in disease predisposition (Horton, et al., 2004; Muller-Hilke, 2008; Shiina, et al., 2004).

1.7 Celiac disease

1.7.1 Factors causing celiac disease

Celiac disease (CD) is a complex inflammatory disorder caused by the interplay between genetic factors mainly HLA-DQ2 and HLA-DQ8 and environmental factors, mainly wheat gluten. Most celiac patients have HLA-DQ2 (HLA-DQA1*0501/HLA-DQB1*02) and minority of the patients do express HLA-DQ8 (HLA-DQA1*0301/HLA-DQB1*0302) (Sollid, 2002). Host genetic factors contribute to the pathogenesis of CD, with high (approx 70% -75%) rate of occurrence among monozygotic twins (Greco, et al., 2002), suggesting strong genetic link to disease. Ingestion of gluten, and related proteins of rye and barley lead to chronic inflammation, injury in small intestine mucosa, nutrient malabsorption, leading to villous atrophy, crypt hyperplasia (proximal part of small intestine) and flattening of mucosa, in celiac disease patients (Alaedini and Green, 2005; Kagnoff, 2005; Sollid, 2002; Sollid and Lundin, 2009). The disease is prevalent among 0,5% of caucasian population, but subjective symptoms are prevalent only among 20-50% individuals, and can be treated by having diet without gluten (Fasano and Catassi, 2001).

1.7.2 Gluten antigen and HLA-DQ mediated presentation

Wheat gluten and related proteins from barley and rye are the disease precipitating factors. Major types of proteins present in gluten are gliadins and glutenins, triggering the disease (Dewar, et al., 2006; Molberg, et al., 2003; van de Wal, et al., 1999). CD can also be activated by hordeins and secalins present in rye and barley, (Vader, et al., 2003) to some extent with oats (Arentz-Hansen, et al., 2004; Hogberg, et al., 2004; Janatuinen, et al., 2002), analogous proteins in millet, sorghum, rice and maize do not activate CD at all (Spaenij-Dekking, et al., 2005).

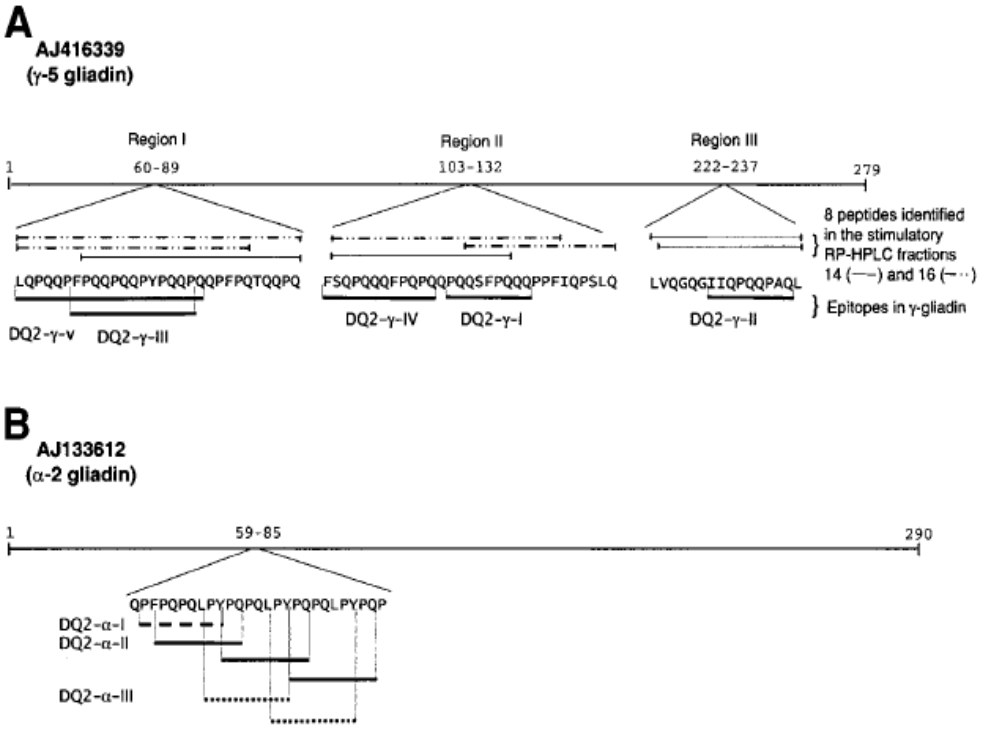

Figure 10: Map of peptide fragments obtained from γ-V gliadin and α-2 gliadin. Figure shows the accumulation of DQ2 epitopes in the gliadin region (Arentz-Hansen, et al., 2002).

In the region of alpha and gamma gliadins, there is enhanced epitope accumulation of DQ2 epitopes with region having high prolines (Arentz-Hansen, et al., 2002). Celiac-lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline, also high glutamines, and this high proline amount makes complete digestion quite difficult and resistant, as human intestinal gastric, brush border and pancreatic enzymes, are deficient in prolyl endopeptidase activity (Hausch, et al., 2002; Shan, et al., 2002). Therefore the local concentration of these high proline and glutamine containing peptides, reaches high levels due to constant accumulation (Shan, et al., 2002; Shan, et al., 2005).
Most importantly under physiological conditions, gluten peptides after degradation, further survive in form of a 33-mer fragment (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) α2-gliadin 56–88. The fragment is highly stable and resistant to enzymatic & proteolytic attack, containing six partly overlapping copies of three DQ2-restricted T cell α epitopes, reacting with tTG (tissue transglutaminase) with much higher affinity than any other known gluten peptide. Fragment has strong inducing effect to gut T cell lines from CD patients. Also this 33-mer fragment has a characteristic type II polyproline helical conformation in solution (Shan, et al., 2002), which is the preferred conformation adopted by ligands bound to class II MHC proteins making binding more preferable (Jardetzky, et al., 1996). Gluten antigens bind to HLA-DQ2 and HLA-DQ8 molecules expressed on APC and present antigens to CD4+ T cells in the lamina propria of the small intestine, thus conferring susceptibility to CD (Johansen, et al., 1996; Kagnoff, 2005; Lundin, et al., 1990; Lundin, et al., 1990; Mazzarella, et al., 2003; Molberg, et al., 1998), or can also modify the T cell repertoire, during thymic T cell development. Small intestine biopsies from the treated CD patients,

when challenged with antigenic gluten peptides leads to activation of lamina propria CD4 + T cells, in *in vitro* settings, only in treated CD patients but not in healthy controls (Halstensen, et al., 1993).

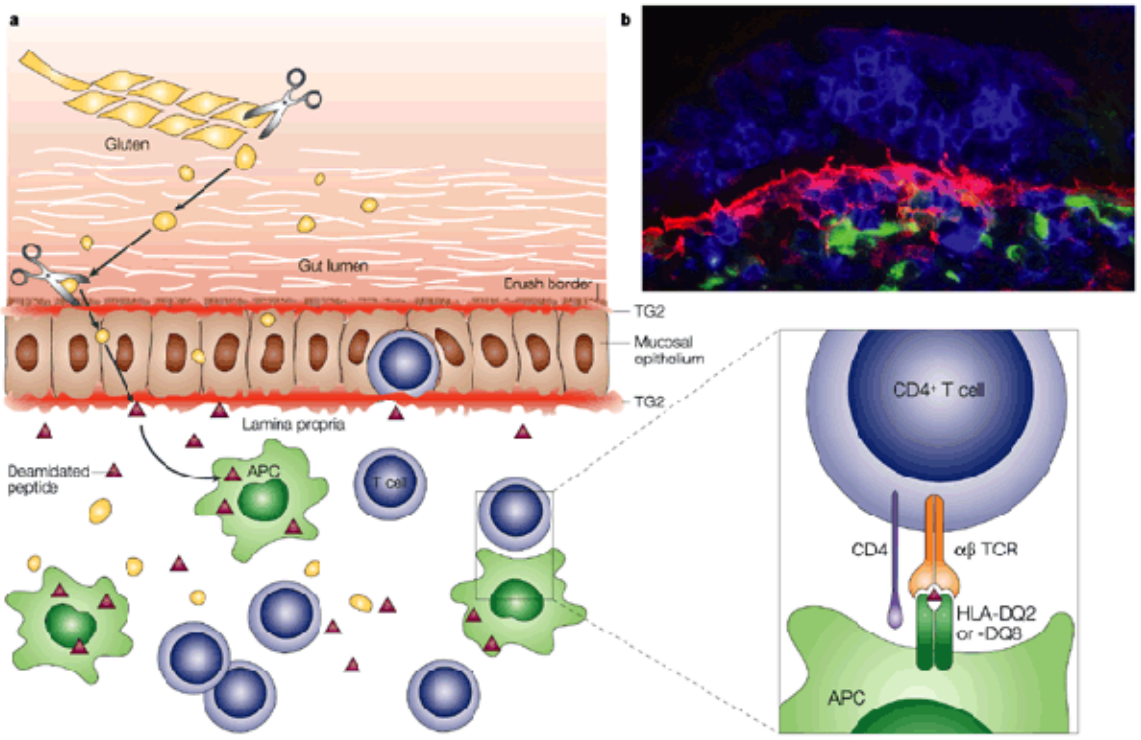

Figure 11: Diagrammatic representation of small intestine lesion of a celiac patient. a) Gluten derived peptides that are resistant to luminal and brush border enzyme processing survive and can be transported across mucosal epithelium. Deamidated gluten peptides bind to HLA-DQ2 or HLA-DQ8 on APC cell surface and get presented to respective T cells. b) Immunofluorescence staining showing TG2 (pink), T cells (purple) and HLA-DQ2 (green) from a section of small intestine of a celiac disease patient. (Sollid, 2002)

Antigenicity of the gluten antigen is further increased by the deamidation of gluten peptides at selective positions by enzyme tissue transglutaminase (TG2) *in situ*, converting neutral glutamine residues to negatively charged glutamic acid which then binds to HLA-DQ2 and HLA-DQ8 more effficietly (Molberg, Ø. NM,1998) (Molberg, Ø,2001 EJI. 31) (van de Wal, Y,1998,JI). Characterization of natural ligand suggest that negative charges can be favoured as all the positions (Stepniak, et al., 2008). Peptide anchor positions of DQ8 (has preference for negative charges at positions P1, P4 & P9 (Godkin, et al., 1997; Kwok, et al., 1996; Lee, et al., 2001) however in case of DQ2 is P4, P6 & P7 (Johansen, et al., 1996; van de Wal, et al., 1996; Vartdal, et al., 1996). Thus incorporation of negative charges makes antigen better DQ binders, and thus better activation of gluten specific T cells in small intestine of CD patients (Molberg, et al., 1997; Molberg, et al., 1998). Partially digested gluten peptides some how in genetically succeptible CD patients may gain access to cross the small intestinal epithelial to reach lamina propria, where these deamidated gluten antigens get presented to CD4+ T cells via HLA-DQ2 or DQ8 on APC (Sollid, 2002). Or by uptake of peptides by DC arms, which can penetrate and sample gut

epithelium (Rescigno, et al., 2001). Additionally these free gluten peptides can co-internalize with TG2, or TG2 linked gluten peptides itself, and then get deamidated inside endosomes and finally get loaded on HLA-DQ2 or DQ8 allele, for presentation to T cells . However normal physiological role of TG2 enzyme is tissue repair and crosslinking of proteins and can catalyze the modification of covalently protein bound glutamine side chains through transamidation and deamidation, in calcium dependent manner (Piper, et al., 2002).

Simple alcohols and small organic compounds have already shown to enhance antigen loading of HLA-DR molecules (Hopner, et al., 2006; Marin-Esteban, et al., 2004). Small molecules have been shown to catalyse the loading of autoantigens on HLA-DR molecule(Marin-Esteban, et al., 2003). Small molecules effecting ligand exchange on HLA-DQ allele have not been shown yet. In celiac disease, gluten derived antigens are generated in the gut and further loaded on HLA-DQ2 molecules. Its known that human gut is full of the bacterial commensals (Ogra, 1999). Therefore it is possible that some biotransformed product produced by commensals may function as MLE. It is already known that dendritic cells sample that gut lumen by penetrating their dendrites (Rescigno, et al., 2001). Thus these bacterial or commensal product might affect, the ligand repertoire of HLA-DQ2.

2 Objectives

Major histocompatibility complex (MHC) molecules are a key element of the cellular immune response as they present peptide antigens for the surveillance by CD4+ T cells. Our group has recently shown that small molecular 'MHC loading enhancer' (MLE) compounds can enhance antigen loading on MHC class II molecules. I focussed my research on following topics:

Development of 'natural like' short peptide derivatives that could function as 'MHC loading enhancer' (MLE). These enhancers may find application in tumor immunotherapies and vaccinations as drug like vaccine *'additive'*.

Experimentally prove MLE induced conformational change in HLA-DR1 protein as the mechanism behind MLE mediated ligand exchange.

To study the role of 'natural like' short peptides with antigen related to celiac disease. They might work as putative environmental risk factors in disease induction by modulating antigen loading on celiac disease linked HLA-DQ2 molecule.

3 Materials and Methods

3.1 Chemicals and Solutions

Chemicals	Company
ANS (8-anilino-1-napthalelesulfonicacid)	Sigma Aldrich US
Avidin-HRP ™ enzyme conjugate	Sigma
β-naphthoyltrifluoroacetone	Fluka
Betaplate Scint	Wallac
3,3-Diaminobenzidine tablets set (DAB- Peroxidase-Substrat)	Sigma Fast ™
DMEM medium	Invitrogen
Dulbecco's PBS	PAA Laboratories GmbH
Ethanol	Roth, Karlsruhe
Fetal calf serum	Roth
L-Glutamine	Invitrogen Karlsruhe
Me_2SO (DMSO)	Carl Roth GmbH, Germany
NaCl	Roth
Na_2HPO4	Roth
NaH_2PO4	Roth
Paraformaldehyde	Roth
p-chlorophenol	Fluka
Pottasium hydrogen pthalate	Roth
Propium Iodide (PI)	Sigma
RPMI 1640 medium	Gibco
Saponin	Calbiochem USA
[6-^{3}H]-Thymidine	Amersham Pharmacia biotech
Triton X-100	Roth
Tris (hydroxymethyl), aminomethane (Tris)	Roth
Tri-n-octylphosphine oxide	Fluka
Trypan blue	Invitrogen
Tween 20	Roth
Vectashield	Vecta labs

3.2 Antibodies

Specificity	Clone	Company
CD86	GL-1	In house production
CD11c	N418	In house production
HLA-DR	MEM-267, MEM-266	Vaclav Horejsi, IMG Prague
HLA-DR	KL-295, KL-304	Lawrence J Stern, Harvard, US
HLA-DR (L243)	L243	In house production
IFN-γ	AN 18.17.24	In house production
IFN-γ	RA-6A2	Ludvig M Sollid, IMMI Oslo
HLA-DQ2	2.12.E11	Ludvig M Sollid, IMMI Oslo
Pan HLA-DQ	SPV-L3	In house production

In house production means that the antibodies were isolated from hybridoma supernatant by Protein A or Protein G columns, as per manufacturers recommendation.

3.3 Peptides

All the peptides were obtained from EMC microcollections GmbH (Tübingen, Germany), unless specified.

Peptide	Sequence	Reference/Company
Biotinylated deamidated 33mer (α2 gliadin 56-88)	Biotin-Ahx-Ahx-LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF	(Shan, et al., 2002)
Biotinylated deamidated γ-II gliadin	Biotin-Aca-Aca-GIIQPEQPAQL	(Vader, et al., 2003)
Biotinylated HA306-318	Biotin-Ahx-Ahx-PKYVKQNTLKLAT	(Lamb, et al., 1982)
Biotinylated-IC106-120 ('CLIP' peptide)	Biotin-Ahx-Ahx-KMRMATPLLMQALPM	(Riberdy, et al., 1992)
human ABL 908-922	KGKLSRLKPAPPPPP	(Gupta, et al., 2008)
Biotinylated human ABL 908-922	Biotin-Ahx-Ahx-KGKLSRLKPAPPPPP	(Gupta, et al., 2008)
Deamidated 33mer (α2 gliadin 56-88)	LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF	(Shan, et al., 2002)
Deamidated γ-II gliadin γ-5 (227-237) with 'E' at 232	GIIQPEQPAQL	(Vader, et al., 2003)
Deamidated γ-IV gliadin γ-5(102 113) 'E' at 106, and 108	FSQPEQEFPQPQ	(Arentz-Hansen, et al., 2002) Peptide library of Ludvig M Sollid, Oslo
HA306-318	PKYVKQNTLKLAT	(Lamb, et al., 1982)
human ABL 908-922	KGKLSRLKPAPPPPP	(Gupta, et al., 2008)
MHC I α 46-60	EPRAPWIEQEGPEYW	(Vartdal, et al., 1996)
NY-ESO-1 89-101	EFYLAMPFATPME	(Chen, et al., 2004)

3.4 Peptide-MLE

All the peptides were obtained from EMC microcollections GmbH (Tübingen, Germany), (Gupta, et al., 2008)

Peptide-MLE	% DMSO for 100mM stock
YR, AR	0
LRMK, YFR, GYV, Ac-AR-NH_2, Ac-ry-NH_2, Ac-rY-NH_2, Ac-Ry-NH_2, Ac-yRNH_2, Ac-Yr-NH_2, Ac-b3hYR-NH_2	10
Ac-FR-NH_2, Ac-YR-NH_2, Ac-LR-NH_2, Ac-ER-NH_2	15
LPKPPKPV	20
Ac-WR-NH_2	25
Ac-VR-NH_2, Ac-MR-NH_2, Ac-IR-NH_2, Ac-RY-NH_2	100

3.5 Soluble MHC class II molecules

MHC class II	Source
Detergent solubilised HLA-DQ2 (HLA-DQA1*0501/HLA-DQB1*02)	Purified by me while working in the lab of Ludvig M Sollid, IMMI Oslo, also provided by Ulrike Juse.
HLA-DR1 (DRB1*0101), β86G HLA-DR1 (HLA-DRB1*0101 (β86G→V) HLA-DR1 (HLA-DRB1*0101 (β86G→Y)	E Coli expression system kindly provided by Andreas Schlundt, FMP Berlin, SF-9 insect expression system in house production, and Baculovirus expression system, in house production, kindly provided by Christoph and Sebastian Guenther.
Soluble HLA-DQ2 (HLA-DQA1*0501/HLA-DQB1*02)	Kindly provided by Ludvig M Sollid, Ulrike Juse, IMMI, Oslo

3.6 Cells

Cell name	Cell type
722.221 (ATCC)	EBV-transformed B cell
721.221-DRb1GFP cells (Gupta, et al., 2008)	EBV-transformed B cell
Bone marrow derived dentritic cells (from HLA-DR1 transgenic mice)	Mouse primary cells
CD 114 (Qiao, et al., 2005)	EBV-transformed B cell
CTLL (ATCC) (Falk, et al., 2002)	IL-2 dependent cell line
EvHA/X5 (Hopner, et al., 2006)	Mouse T cell hybridoma
HTR (Falk, et al., 2000)	EBV-transformed B cell
L929 HLA-DR1 (DRB1*0101), L929 HLA-DR1 (HLA-DRB1*0101 (β86G→V) HLA-DR1 (HLA-DRB1*0101 (β86G→Y) (Hopner, et al., 2006)	Mouse fibroblast
PD2 (Falk, et al., 2000)	Human CD4+ T cell line
SaABL/G2 (Gupta, et al., 2008)	Mouse T cell hybridoma
TCC 430.1.112 (Arentz-Hansen, et al., 2002)	Human CD4+ T cell clone derived from celiac patient biopsies
TCC 493.3.4.33 T(Arentz-Hansen, et al., 2000)	
TCC Has.1.41 (Qiao, et al., 2005)	
9023 VAVY (distributed by X^{th} international histocompatibility workshop)	EBV-transformed B cell

3.7 Buffers

Buffer	Composition
Bicarbonate antibody coating buffer	100mM $NaHCO_3$ pH 8.3 in water
Citrate Buffer (1X) (HLA-DQ2 Binding buffer)	25mM citric acid, 50mM Na_2HPO_4 pH 7.2
DELFIA,Eu^{+3} labeled streptavidin	Perkin Elmer
DMEM medium	DMEM 1640 with 5% fetal calf serum (FCS), 1 mM Pyruvate, 2 mM L-Glutamine, 10 mM N-2Hydroxyethyl-N´2´piperazinethylsulfonate, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 1 mM non essential aminoacid (NEA), 0,05 mM β-Mercaptoethanol
ELISPOT wash buffer	PBS 0.05% tween 20
Enhancer solution	15µM b-naphthoyltrifluoroacetone (Fluka), 50µM tri-n-octylphosphine oxide (Fluka), 6.8mM pottasium hydrogen pthalate (Roth), 100mM acetic acid, 0.1% triton X-100 (Roth)
Erythrocyte lysis buffer	10mM Tris-HCl, 0.165M NH4Cl pH7.2
FACS buffer	PBS 2% fetal calf serum
Fixation buffer	3.7% formaldehyde in PBS pH 7,2
RPMI medium	RPMI1640 with 10% fetal calf serum (FCS), 1 mM Pyruvate, 2 mM L-Glutamine, 10 mM N-2Hydroxyethyl-N´2´piperazinethylsulfonate, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 1 mM non essential aminoacid (NEA), 0,05 mM β-Mercaptoethanol
Saponin buffer	PBS, 0.5%(w/v) saponin, 5% foetal calf serum
Wash Buffer	PBS with 0.05% Tween -20

3.8 Instruments

Instrument	Company
384 well white plate (low binding)	Costar
384 well black plate	Nunc
Bath sonicator	Sonorex, RK100H, Bandelin (Berlin,Germany)
Centrifuge, Megafuge 3.OR	Herareus Instruments GmbH (Berlin, Germany)
ELISPOT multiscreen HTS 96 well filtration plate	Millipore
ELISPOT Reader S4 core analyzer	Cellular Technology Ltd. (Cleveland, USA)
FACS Calibur	BD Biosciences (San Jose, USA)
Harvester	Tomtec (New Heaven, USA)
Incubators (5%, 10%)	Binder
Laminar hood	ICN
Leica Sp5 confocal microscope	Leica microsystems (Germany)
Luminescence Spectrometer LS 50B	Perkin Elmer
Luminescence Spectrometer LS 50B	Perkin Elmer
PCR thermocycler	Eppendorf (Germany)
pH meter	Ino lab
Quartz cuvette for fluorescence experiments	Hellma type no. 105.251-QS/center 8.5mm
Simple cell culture microscope	Olympus CK40
Sorvall RC3C plus	Beckmann (USA)
Table centrifuge	Eppendorf (Germany)
[^{3}H]-Thymidine Reader 1450 microbeta Wallac Jet liquid scintillation and luminescence counter	Wallac (Turku, FIN), Perkin Elmer
Victor 3V reader, 1420 multilabel counter	Perkin Elmer
Weighing balance	Metter Toledo
Zeiss LSM 510 confocal microscope	Carl Zeiss MicroImaging Inc. (Germany)

3.9 Softwares

Software	Company
Adobe® Acrobat professional ® 8.1.2, Abode® Illustrator® CS3 13.0.2, Adobe® Photoshop® CS3 10.0.1	Adobe systems incorporated, USA
BD FacsDiva	BD Biosciences (San Jose, USA)
CellQuest pro	BD Biosciences (San Jose, USA)
Chemoffice Ultra	Cambridge software
Filemaker Pro 7.0	Filemaker Inc. USA
ImageJ 1.38X	National Institute of Health, USA
Immunospot® 4.0 Professional software	Cellular technology Ltd. (Cleveland, USA)
Microsoft office	Microsoft Redmont, USA
Sigmaplot version 9.0	Systat software Inc.
Zeiss LSM 510	Carl Zeiss, Germany

3.10 Enzyme-Linked Immunosorbant Assay (ELISA)

384 Nunc maxisorp plates were coated with L243 (α-HLA-DR capture antibody-1 µg/ml made in sodium bicarbonate coating buffer). 50µl/well of the 1µg/ml, L243 antibody solution was incubated at 37°C/ 2h. The plates were then washed two times with wash buffer. Plates were then blocked with PBS+2% BSA at 37°C/ 2h. BSA blocked plates were washed 4 times with wash buffer, and the diluted loading reaction mixture was splitted into two and transferred to the washed plates manually, by 12 channel pipette. Final volume to be 50µl/well. Plates were then kept for binding at 4°C/1h 30min. Plates were washed 6 times with wash buffer. $Eu+^{3}$-labelled streptavidin was diluted to 1:2500 in PBS 1% dialyzed BSA, and 50µl/well was added to the ELISA plates and incubated for 30min at room temperature. After incubation plates were finally washed 8 times, and 75µl per well of fluorescence enhancing enhancer solution was added to the plates and kept for 5 min. Finally the Eu^{3+} fluorescence was measured in a Victor fluorescence reader (Wallac) using the time-resolved mode at an excitation wavelength of 340 nm and an emission wavelength of 614 nm. Results are expressed as counts per minute (cpm).

3.11 Labelling of HLA-DR molecules with biotin

HLA-DR proteins were labelled using EZ-Link^{R} NHS-Biotin reagents (Pierce Biotechnology). Briefly 10mM biotin stock was prepared in dimethyl sulfoxide (DMSO). 20 fold molar excess of biotin was added to the HLA-DR protein, as per manufacturers recommendation. The reaction was incubated on shaker kept at 4°C/ 2h. Subsequently unbound free biotin was removed by spin column, by repeated spinning and adding sterile PBS pH 7.2. Finally concentration of protein was determined by Bradford method, and utilized for the experiments involving conformational sensitive antibody.

3.12 ELISpot assay

ELISpot plates were prewet with 15µl/well of 70% methanol (v/v in Milli Q water) for one minute with subsequent washing with 150µl of sterile PBS (Phosphate buffer saline). Overnight coating of plates were done with 7.5µg/ml anti-IFN-γ antibody (clone AN18.1724) in sterile PBS/ 4°C or 37°C/ 2h. Washing of plates was done 2 times with 5min incubation between washing steps. Blocking of plates was done with 200µl of DMEM 5% FCS, at 37°C/ 2h. After removal of block medium 200µl of lymph or spleen cells with antigen and dipeptides were transferred into the plate. As a positive control cells were also stimulated with anti-mCD3 (9 µg/ml) und anti-mCD28 (5 µg/ml), and as negative control cells were kept without stimulation. Plates were incubated at 37°C/ 24h/ 10% CO_2 incubator. Washing of plates was done 6 times with PBS/0.05% tween. 100µl/ well of secondary biotinylated anti-INF-γ antibody (clone R4-6A2) was added and incubated for 2h/37°C /10% CO_2 incubator. Washing of plates was done 6 times with PBS/0,05% tween, 1:1000 dilution of Avidin-HRP ^{TM} enzyme conjugate (2µg/ml), was done and 100µl/well was added and incubated for 1h/room temperature. Washing of plates was done 3 times with PBS/0,05% tween and then 3 times with PBS. DAB (3,3 Diaminobenzidine) substrate tablets were mixed in MilliQ water (eg. 4+4 tablets /5ml water) as per manufacturere protocol, and filtered by using 0,45µ filter. 50µl/well of

filtered solution was added. Spots were developed by incubating with substrate for 3-4min. Extensive washing was done, under running water. Single well tray was removed and excess substrate was also removed from membrane, by tapping on paper towel. Plates were kept overnight in dark for drying. Spots were counted using immunospot reader.

3.13 Confocal laser scanning microscopy

Confocal microscopy experiments were done with 721.221 cells expressing HLA-DR-GFP fusion protein (721.221-DRaGFP). Briefly 10 x 10^4 721.221-DRaGFP cells/well were incubated with 20µg/ml of biotinylated HA306-318 peptide in presence of 2.5mM of peptide-MLE Ac-FR-NH_2 (4h, 37 °C, DMEM, 5% FCS, 96 well V-bottom plates). Cells were then washed 3 times with 200µl PBS. Cells were resuspended by 120µl of RPMI medium or PBS. 120µl of the cell suspension were then dropped carefully on poly-L-lysine (sigma) precoated coverslips, kept inside 12 or 24 well plate so to make all cells settle on coverslips avoiding overflowing of cells in wells. Incubation was done for 30-45min at 37 °C. To confirm if cells have sticked to the coverslips, plate was checked on microscope. Fixation of cells were done by adding 2ml of 3,7% formaldehyde in PBS and incubating them for 10-15min at RT. Washing was done with wash buffer (PBS+ 0.02% tween 20) 3 times. Blocking was done by adding 2ml of PBS+ 2% BSA+ 0.02% tween-20 at room temperature for 30-40min. Excess block buffer was removed carefully, and 200µl/well of diluted Streptavidin-Cy5, was added just to cover the coverslips for staining. Staining was done at room temperature for 35-40min.Washing was done with PBS+0.02% tween-20 for 4-5 times. Coverslips were taken out via forcep from the 12 / 24 well plate and dipped in MilliQ water, then was touched on filter paper for removing excess water. A drop of mounting media vectashield was placed on mounting slides, and then coverslip was mounted on the slide in inverted fashion (so that cells are on innerside). After drying edges of the coverslip were sealed by applying nailpolish, waited for 15 min for drying. Fixed cell microscopy was performed on a Zeiss LSM510Meta confocal setup mounted on an Axiovert 200M inverted microscope using a 63x phase contrast plan-apochromat oil objective numerical aperture 1.4. For all images, the acquisition was done with main beam splitter HFT UV/488/543/633 and the specific parameter for each fluorophores were, GFP excitation at 488 nm and detection with 500-530 nm BP (band pass filter), Cy5 excitation at 633 nm and detection with 650 nm LP (long pass filter). Image acquisition was done sequentially. The images were analyzed and arranged using Adobe photoshop CS3 and ImageJ software.

3.14 Fluorescence activated cell sorting (FACS)

Flow cytometry experiments were done to estimate the amount of peptide loading on cell surface MHC molecules. Briefly APC loading were carried out with either L929 fibroblast and 722.221 cells expressing HLA-DR, or VAVY cells expressing HLA-DQ2. Staining of antigen loaded cells was done with FITC (fluorosceinisothiocyanate) fluorophore conjugated antibodies to HLA-DR, HLA-DQ or streptavidin phycoerythrin (SA-PE). Life gated (propium iodide negative) cells were analyzed. Amount of cell surface MHC/petide complex were estimated by geometrical mean values. Data were plotted using sigma plot to generate dose response curve.

3.15 Cell culture

3.15.1 Maintainence of antigen presenting cells (APC)

L929 cells were expressing HLA-DR1 were maintained using DMEM 5% FCS, in 10% CO_2 incubator, in a T flask. Splitting of the cells was done when the cells were 70-80% confluent. Trypsinization was done to detach the adherent cells. EBV transformed B lymphoblastoid cell lines like 722.221, 721.221-DRb1GFP cells, HTR, VAVY and CD114 cells were cultured in RPMI 10% FCS in 5% CO_2 incubator. Splitting of the cells were done every two days.

3.15.2 Maintainence of T cells

The T cell hybridomas EvHAX5 and SaABL/G2 were cultured in DMEM 5% FCS/ 10% CO2 incubator, in a T flask. Human T cell line PD2 were cultured in RPMI 7% human serum 5% CO2, in a 96 well plate. Human T cell clones obtained from celiac disease patients TCC 430.1.112, TCC 493.3.4.33, TCC Has.1.41 were cultured in RPMI 10% human serum (HS) 5% CO2. The in vitro culture, cloning and characterisation of these HLA-DQ2 restricted T cell clones have been described elsewhere (Molberg, 2000). The cells were counted and utilized for carrying out T cell assays.

3.15.3 Isolation and *in vitro* maturation of dendritic cells (DC)

Mouse bone marrow progenitor cells are cultured in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) to stimulate proliferation and maturation of dendritic cells (DC) (Lutz, et al., 1999). Femur and tibia bones from the mice were removed in one whole piece, eliminating as much as possible the muscular tissue. Under sterile conditions removed bones were kept in a six well plate with 80% ethanol for 10sec then into the well containing medium RPMI+10% FCS or a petridish containing medium. This process was repeated twice. Both the ends of bone were cut and then bone marrow was flushed with medium using 10ml syringe (needle 20G x 1 ½) into a new petridish containing medium, in sterile conditions. This process was repeated with other bones too. Single cell suspension was prepared by passing the marrows through the needle, and reflushing on the petridish to resuspend clumps of marrow if any. The marrow suspension was resuspended and pelleted for 5 min/ 1000rpm.
At day 0 DC precursor cells get adhered. Adherent marrow pellet was resuspended in RPMI +10% FCS medium containing 15ng/ml of GM-CSF. 2 ml of cell suspension was seeded in a 24 well plate or a 12 well culture dish respectively. On day 1 medium was changed. Culture medium was mixed gently 3-4 times using pipette to resuspend the non adherent cells, and then medium was removed completely. 2 ml of new medium with 15ng/ml GM-CSF was added. On day 3: Suspension clusters consisting of granulocytes are seen. Culture medium was mixed gently 3-4 times using pipette to resuspend the non adherent cells, and then medium was removed completely. 1 ml of new medium with 15ng/ml GM-CSF was added. On day 4-7 small clusters of DC are seen with maximum proliferation rate from day 4 to 5, with no more DC cluster detectable around day 7-9. On day 5, 1-2 ml of more medium containing GM-CSF was added, without removing the previous medium (as immature DCs are non adherent). Cell clusters were disaggregated by

resuspending gently. On day 7: immature DC are seen (If DC purity not >80% may be problems with GM-CSF quality or quantity). Adherent as well as non adherent DC were recovered by gently pipeting the own media of each well over the adherent cell layer. Cells were centrifuged at 1000rpm for 10min. To induce DC maturation, cells were put back into culture for 2 days (maximum), in same plate with new medium containing, 10ng/ml LPS. Day 9: Mature Dendritic cells were harvested and checked for maturation status. Maturation of bone marrow derived DC was confirmed by staining with anti-mouse CD86-FITC, also anti-mouse CD11c-APC staining was done to confirm DC population.

3.16 CTLL assay

CTLL (ATCC) is an IL-2 dependent cell line, which grows in the presence of IL-2. This is an indirect assay for the estimation of the IL-2 production. Assay was performed to estimate the amount of IL-2 released with T cell activation, as mentioned (Falk, et al., 2002)
Briefly 30µl of APC: T cell hybridoma withdrawn supernatants were used for the assay. CTLL cells were washed 2 times with DMEM 5% FCS and then counted to finally adjust cell count $1X10^6$ cells/ml. 50µl (50,000 cells/well) of the cell suspension was added to 30µl of supernatant, and then plate was kept at 37 °C/12-16h in 10% CO_2 incubator. Then thymidine was added to measure the IL-2 release, during T cell activation.

3.17 [H^3] thymidine assay

Proliferation of T cells was measured by thymidine incorporation using radiolabelled nucleotide. Dilution of [H^3] Thymidine (stock: 1µCurie/ml) was done in a way so to attain final concentration 0.65µCurie/well. 50µl/well of diluted stock was added. Plates were then kept for 12h at 37°C/5% incubator. Finally cells were harvested and transferred onto glass fiber filter mat using automated cell harvester. Filter mats were allowed to dry for 1h/room temperature, with subsequent insertion into polybag. Betaplate Scint liquid was added, and polybags were then sealed. Plates were then measured in microbeta wallac scintillation counter. Counts obtained were utilized to calculate either proliferation (Human T cell lines or clones) or IL-2 release (Mouse T cell hybridoma).

3.18 Peptide loading of soluble MHC molecules

3.18.1 Loading of 'empty' HLA-DR molecules

To confirm the effect that peptide-MLE can catalyze the loading of peptide antigens on soluble HLA-DR molecules, peptide loading assay was performed. The loading experiment is done as described (Marin-Esteban, et al., 2004). For loading experiments 100nM, HLA-DR1 wt (β86G) protein was incubated with 40µg/ml of biotinylated HA306-318 peptide at 37°C /1h. For loading experiments involving Biot-ABL908-922 peptide, 1.5µg/ml of ABL908-922 peptide was incubated with HLA-DR wt (β86G & HLA-DR1 mut (β86G→V) and 0.2µg/ml peptide was incubated with HLA-DR tyrosine mutant (β86G→Y) in presence and absence of MLE at 37°C/1h. All the *in vitro* loading experiments were done in PBS, with 1% dialyzed BSA (DBSA) and volume of loading reaction was 20µl. After 1h incubation the loading reaction was diluted to 1:6 by adding

ice cold 1% DBSA, to make the final volume to 120µl. 50µl per well was transferred in duplicates to ELISA plates, and the measurement and assay was carried out using ELISA protocol as mentioned in section 3.10.

3.18.2 Peptide loading of 'empty' HLA-DQ2 molecules

To confirm the effect that peptide MLE can catalyze the loading of gluten derived antigens on soluble HLA-DR molecules, peptide loading assay was performed.
Briefly 100nM of recombinant HLA-DQ2 or 100nM of detergent solubilised HLA-DQ2, were incubated with either 200nM of deamidated 33mer or 5µg/ml of deamidated γ-II gluten derived gliadin antigen at 37°C in DQ2 binding buffer pH 7.2/16h. Fold enhancement in MHC/peptide complex was determined by ELISA protocol as mentioned in section 3.10, except the coating of plates were done by HLA-DQ2 specific monoclonal antibody 2.12.E11 or pan HLA-DQ reactive antibody SPV-L3.

3.19 Ligand-exchange of soluble HLA-DR molecules

The ligand exchange experiment were done as described (Hopner, et al., 2006). *In vitro* ligand exchange experiments were carried out by loading 1.5 µM of HLA-DR1 with 50µg/ml of biotinylated IC106-120 for 18-20 hrs in presence of 5% ethanol in PBS with 1% DBSA, pH 7.4. After 20 hrs the HLA-DR1: IC complex was diluted to 1:15 and was incubated with 200µg/ml high affine HA306-318 peptide in presence and absence of 10mM dipeptide. The reaction was set up in a 96 well V bottom eppendorf plate, at 37 °C in a thermocycler. This reaction was done in time dependent kinetic fashion with timely withdrawl of 10µl of samples, using a 12 channel pipette. 10µl samples were immediately added to 110µl ice cold PBS+1% BSA, in order to dilute and slow down the peptide loading reaction. Subsequently the samples were frozen at -20°C. The amount of HLA-DR/biotin-IC106-120 remaining after incubation was determined by ELISA as mentioned in section 3.10. (Marin-Esteban, et al., 2004).

3.20 Calculation of the 'catalytic rate enhancement'

The 'catalytic rate enhancement' coefficient was determined in loading assays with 100 nM soluble HLA-DR1 and 50 µg/ml HA306-318 with titrated amounts of the catalytic peptide-MLE (1 h, 37°C, pH 7.4). A curve fit was carried out by a hyperbola regression ($f(x) = ax/(b+x)$) using the Sigmaplot Version 9.0 software (Systat Software Inc.) and the coefficient was determined by forming the average of the starting slope (a/b) of 2–4 independent experiments (Gupta, et al., 2008).

3.21 Antigen loading of the cell surface MHC molecules

The cell surface HLA-DR1 loading experiment is done as described (Hopner, et al., 2006). APC were counted and cell count was adjusted to 2 x 10^6 cells/ml. Peptide-MLE or chemical-MLE compounds were diluted and titrated to get desired concentration in a V bottom plate with volume reaching 50µl/well. Now 50µl of biotinylated antigenic peptide was added in the required concentration. Finally 50µl of cell suspension was added to the plate in order to get 100,000 cells/well with final reaction volume of 150µl. This loading

reaction was incubated at 37 °C/ CO_2 incubator for 4h for HLA-DR1 expressing APC and 24h for HLA-DQ2 expressing APC. The cells were then washed 2 times with PBS+1% FCS, with subsequent tapping on paper towels to remove any unbound peptide. Now cells were vortexed and stained with 35µl/well of Streptavidin-PE for 30 min at 4°C.After incubation cells were again washed 2 times with PBS+1% FCS, with subsequent tapping on paper towels, to remove unbound stain. Cells were then resuspended in 100µl of propidium iodide (PI) (8µg/ml) and transferred to the FACS tube. Additional 100 µl of FACS buffer (PBS with 1% FCS) was added to the FACS tube for a final cell suspension for 200µl. FACS tube were kept at ice during measurement. Live gated cells (PI negative) were analyzed by FACS on a FACS Calibur instrument, to measure the amount of PE signal.The amount of PE signal correlated directly to the cell surface MHC: peptide complex. Results were finally expressed as geometric mean.

3.22 T cell assays

T cell assays were carried out to confirm if MLE catalyzed antigen loading can also be translated into amplification of antigen specific CD4+ T cell immune response.
The T cell assays were done using two protocols 'pulse wash' and 'permanent'. For carrying out the experiments with human T cell lines or T cell clones, APC were pre-irradiated with 75Gy, for 500 second, for HLA-DQ2 assays and 520 sec (60, 66 Gy or 6066,66 Rad), for HLA-DR assays.

3.22.1 Pulse wash

200µl of 0.25 x10^6 T cells/ml (50,000 cells/well) were added to the preloaded washed APC plate. For antigenic presentation the APC-T cell mixture was incubated for 24h/ DMEM+5% FCS in 96-well U-bottom plates (Marin-Esteban, et al., 2004). After incubation, the plate was centrifuged at 1300rpm/3min, and then 30µl supernatant was withdrawn for the assay and 150µl supernatant was frozen at 80^{o}C as backup. T cell supernatant were used to challenge CTLL line, in a CTLL assay, as mentioned in section 3.16, and then [$_1H^3$] thymidine assay was carried out to determine IL-2 release, as mentioned in section 3.17.

3.22.2 Permanent exposure

In Permanent exposure T cell assays APC, antigen, MLE and T cells stayed together for 48h without any pulse wash (Gupta, et al., 2008). IL-2 release due to T cell activation was measured by CTLL assay as mentioned in section 3.16 and then [H^3] thymidine assay. T cell proliferation was measured directly by adding thymidine to APC/T cell mixture and performing thymidine assay as mentioned in section 3.17.

3.23 ANS binding measurements

Fluorescence binding experiments with HLA-DR protein was performed with environment sensitive ANS dye (8-aninilino-1-napthalenesulfonic acid). Briefly 1µM of HLA-DR protein obtained from E.coli expression system was incubated with 100µM of ANS dye in presence and absence of MLE compounds in PBS (pH 7.25) /2h/37^{o}C. The samples were measured in luminescence spectrometer operated at slit width 8nm for both excitation and emission. Samples were measured using 70µl quartz cuvette with 3mm path length in a temperature controlled cell holder set at 37°C. Excitation of samples were done at 350nm and subsequent emission spectra was recorded by scanning the samples from 400-600nm. Scanning speed was adjusted to 500. Data recorded was an average of minimum 6 sample scannings. The data obtained was plotted using sigma plot.

3.24 Intrinsic tryptophan fluorescence measurements

Briefly 800nM of HLA-DR protein was incubated in presence and absence of MLE compounds for 2h/37^{o}C in PBS pH 7.25. Subsequently emission was monitored from 310nm to 410 nm after excitation of samples at 295nm, in a luminescence spectrometer. Measurements were carried out in temperature controlled cell holder (37°C) using 70µl quartz cuvette with 3mm path length. Scanning speed was adjusted to 350, excitation slit width used was 6nm. Recording of data was done after taking average of atleast 5 individual scans.The data generated was plotted using sigma plot software.

3.25 Probing with conformational specific antibodies

To determine the conformational transitions with conformation specific antibodies ELISA was carried out with MEM-267, MEM-266 and KL-295 antibodies. Briefly antibodies were diluted in sodium bicarbonate coating buffer pH8.3 to achieve the final concentration of 5µg/ml. Briefly biotinylated HLA-DR protein was incubated with HA306-318 peptide or ABL908-922 peptide in presence and absence of MLE compounds, in PBS without FCS. Enhanced MEM reactivity to HLA-DR protein in presence of MLE compounds were quantitated by ELISA protocol as mentioned before in section 3.10.

3.26 MLE effect by conformation specific antibodies

Catalytic effect of conformational specific antibodies (MEM-266, MEM-267) were done by incubating 100nM HLA-DR with biotinylated ABL908-922 peptide in presence of titrated amount of antibodies. Enhanced MHC/peptide complex was detected by L243 antibody coated plates utilising the ELISA protocol as mentioned before in section 3.10.

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4 Results

4.1 Anchor side chains of short peptide fragments trigger ligand exchange of class II MHC proteins.

4.1.1 Rationally designed short peptides show "MLE" activity

By systematic screen of 20,000 library compounds we have previously identified chemical compounds that can enhance the antigen loading on MHC class II proteins by targeting polymorphic Pocket 1. The activity of these compounds strictly correlated with depth of Pocket 1. Thus at least for these 'MHC loading enhancer' (MLE) compounds, Pocket 1 seems to play important role, in mediating ligand exchange (Hopner, et al., 2006). Pocket 1 is in the peptide binding groove, and is located close to the N terminal side of peptide ligand. Pocket-1 accomodates the side chain of a key anchor residue of the peptide ligand. As evident from the crystal structure in the case of HLA-DR1 and HA306-318 (Haemagglutinin antigen from influenza), P1-pocket accommodates tyrosine side chain **(Figure 1)**.

Peptide/HLA-DR1 complex

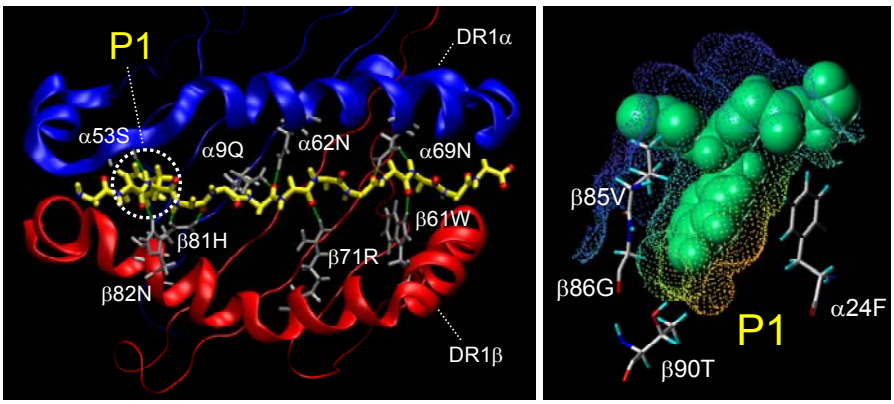

Figure 1: Crystal structure of HLA-DR1 with HA306-318 peptide: Left panel: top view shows the location of pocket 1, with HA peptide bound to HLA-DR1 protein, with H-bond network, that stabilize the peptide, in the binding groove. Only the α1 and β1-domain of MHC molecule are depicted. α1 shown by blue ribbon and the β1-domain of the MHC molecule is depicted by red ribbon. Location of P1 pocket is depicted by dotted circle. Peptide backbone of HA306-318 ligand is enumerated by yellow colour and MHC residues forming H bond interaction with backbone are labelled with grey. Right panel: side view of a P1 pocket showing insertion of tyrosine anchor side chain, of HA peptide. Pocket 1 surface is shown in yellow colour, MHC residues forming the P1 pocket are indicated, with peptide shown in green space fill mode. Pictures are based on the crystal structure of HA306-318/HLA-DR1 (pdb:1dlh) (Gupta, et al., 2008; Stern, et al., 1994)

From the previous results it was concluded that the ligand exchange was triggered by filling pocket 1. Thus it was assumed that MLE effect can also be achieved by the side chain of small peptides that may fill pocket 1. To validate this rationale, simple short peptides, mainly dipeptides were designed, that may fill pocket 1. Experiments were carried out by HA306-318 peptide, an influenza epitope which binds to HLA-DR1 with high affinity. To determine the loading rate soluble HLA-DR1 protein was incubated with biotinylated HA306-318, in presence of dipeptides. While free amino acids did not exhibit any MLE effect (data not shown), simple dipeptide Tyr-Arg (YR) accelerated the MHC loading with HA306-318 peptide in a dose dependent manner (Figure 2). No effect was observed with the Ala-Arg (AR) dipeptide lacking the aromatic anchor side chain, indicating that the replacement of tyrosine by alanine completely abrogated the MLE-effect.

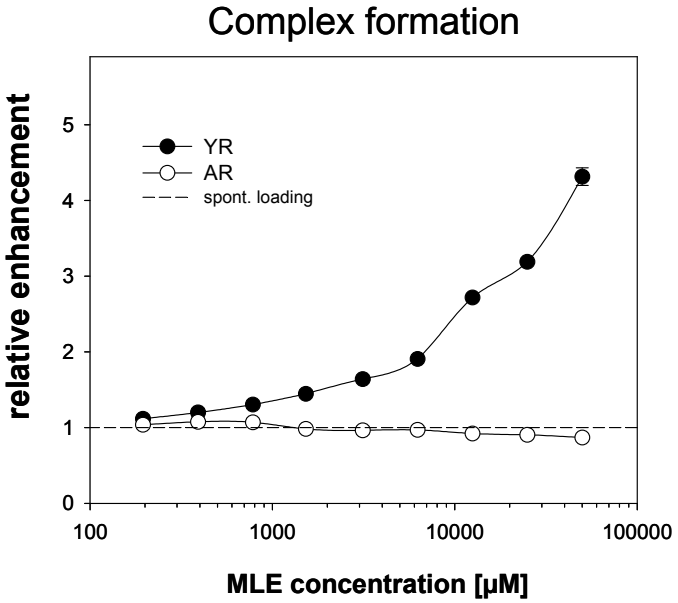

Figure 2: Short peptide side chain amino acid anchors can catalyze the formation MHC/ligand complex. Impact of side chains of the dipeptide in forming antigen-complexes. The effect of short peptide fragments on formation of MHC/ligand complex with HA306-318 peptide and soluble HLA-DR1 was determined. The antigen loading experiment was carried in the absence of dipeptide (dashed line) or presence of titrated amounts of dipeptides. Tyrosine-Arginine (YR shown by filled circle) or Alanine-Arginine (AR shown by open circle). Formation of complex was determined by ELISA. Relative enhancemnent (y-axis) suggests fold increase of MHC/peptide complex formation in presence of short peptide fragments, in comparison to enhancement in absence of dipeptides. Concentration of dipeptides are shown in x-axis (Gupta, et al., 2008).

4.1.2 Hydrogen bond forming groups enhance MLE activity

Although the designed short peptides showed MLE effect, the catalytic activity was observed only at higher concentrations. Peptide binding to MHC molecules is stabilized by hydrogen bond (-H) networks formed with peptide backbone and conserved MHC residues, apart from side chain anchor residue interactions (Stern, et al., 1994). Considerable amount of H-bond are formed around the vicinity of P1-pocket (Figure 1). Most of these would be lost if free dipeptides are used. This could be recovered if acetyl- and amide- groups were introduced to the N- and C-termini of the dipeptides. This would stabilize the dipeptide by maximizing the number of H bonds, and would position the peptidic side chain into P1 pocket. Computational docking of Ac-YR-NH_2 into the P1-pocket of HLA-DR1 suggests that as much as five of the conserved H-bonds can be formed with this minimal peptide (Figure 3a). Dipeptides having H-bonds interacting modifications were used to load biotinylated HA306-318 antigen on HLA-DR1. The MLE activity of Ac-YR-NH_2 was enhanced to nearly 10 fold, as compared to free YR dipeptide (Figure 3b). Partial effects were also observed with Ac-YR and YR-NH_2, as compared to free YR. Ac-AR-NH_2, lacking aromatic side chain was completely inactive.

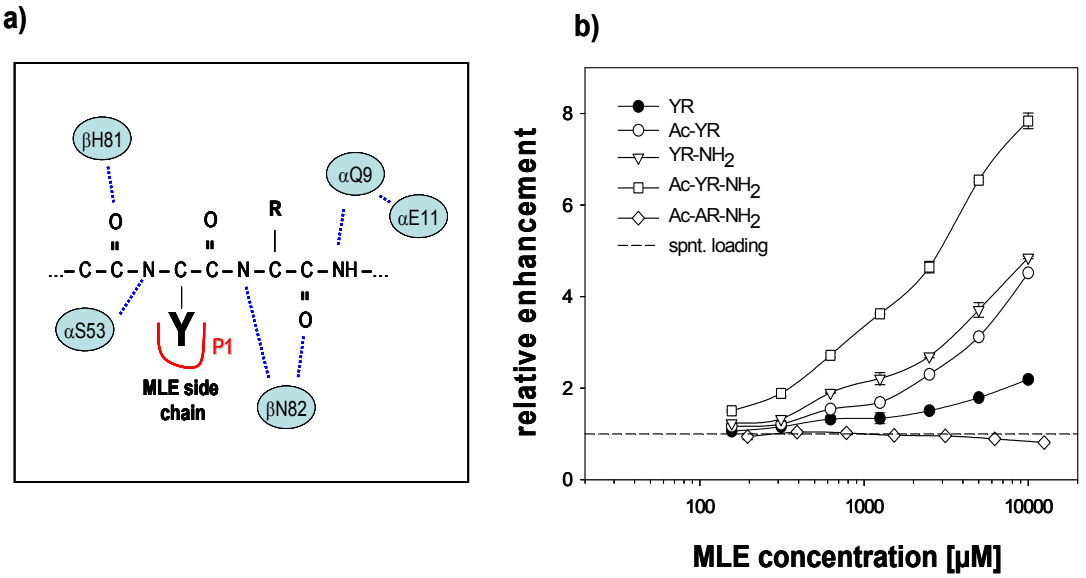

Figure 3: Additional H-bond interactions increase MLE activity. a) A schematic view of H-bond interactions between the peptide backbone and residues within the MHC binding site are shown. H-bonds are depicted as dashed blue lines; residues of the MHC binding site acting as H-bond donor/receptor are indicated by blue elliptic circles. The anchor amino acid (Y) acting as MLE side chain is indicated. H-bond interactions are based on the crystal structure of HA306-318/HLA-DR1 (pdb: 1dlh). b) Impact of the H-bond network on the catalytic activity dipeptides. To provide additional interaction partners for the natural H-bond network N-terminal acetylation and C-terminal amidation were introduced to the YR dipeptide. The effect of these modifications on the catalytic activity is shown for the complex formation between HA306-318 and HLA-DR1. The antigen loading experiment were carried out in the presence of titrated amounts of YR (filled circle), Ac-YR (open circle), YR-NH_2 (open triangle down), Ac-YR-NH_2 (open square) and as control Ac-AR-NH_2 (open diamond). The experiment conditions were same

as mentioned in Fig.2. The spontaneous load in the absence of catalysts is indicated by a dashed line (Gupta, et al., 2008).

4.1.3 Dipeptides show *'drug like'* stereospecificity

To confirm the stereospecific effect of dipeptides towards HLA-DR1, dipeptide sets with D-enantiomers (Ac-yr-NH_2) were generated. Their capability to form HLA-DR1/HA complex was validated in loading assay. In contrast to the L-enantiomer (Ac-YR-NH_2), all the dipeptide generated with D-enantiomer were completely inactive. This applied to the retro inverse type, Ac-ry-NH_2 (Figure 4b). The effect indicates strict stereospecific nature of dipeptides for MLE effect. Also to confirm the importance of natural peptidic structure, a dipeptide analogue of Ac-YR-NH_2 was used, in which tyrosine was replaced by β-homotyrosine (b3hY). Introducing β-homotyrosine in place of tyrosine, resulted in increase in distance between side chains by an additional –CH_2 group. This resulted in totally abrogation of MLE activity (Figure 4a). Thus even a single shift in the backbone structure by one carbon unit destroys MLE activity. This strongly supports the strict structural requirement for the peptide-MLE.

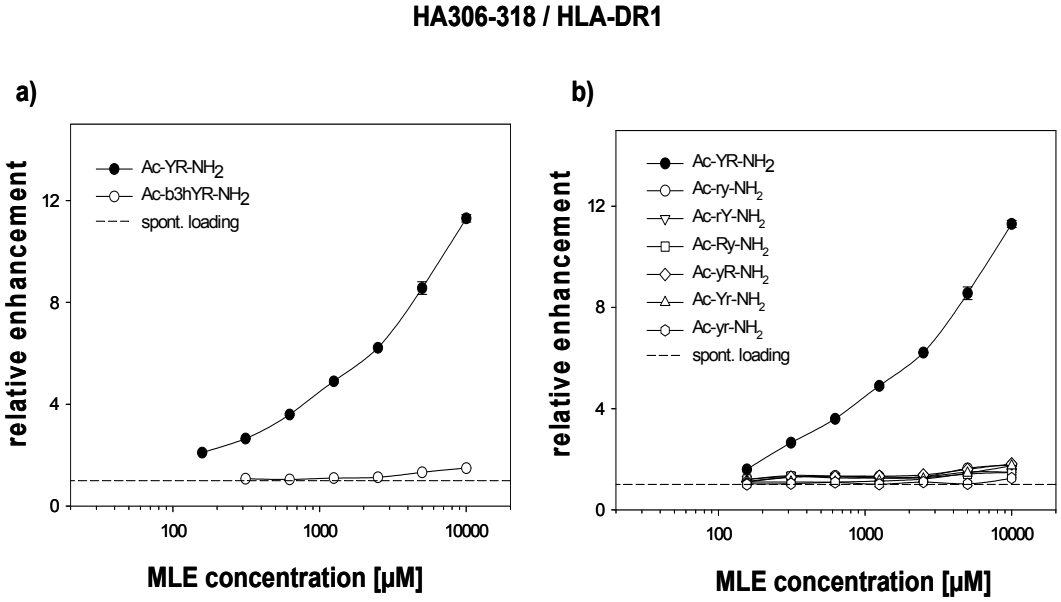

Figure 4: Dipeptides show drug like stereospecificity and stereoselectivity. a)Left panel: Effect of extended side chain spacing. A dipeptide derivative was used in which side chain spacing was extended by one –CH2 group residue, by using L-β-homotyrosine (Ac-β3hYR-NH_2; open circle), instead of tyrosine (Ac-YR-NH_2; filled circle).b) Right panel: Influence of D-amino acids. MHC: antigen complex formation was carried out in the presence of titrated amount of Ac-YR-NH_2 (filled circle), Ac-ry-NH_2 (open circle), Ac-rY-NH_2 (open triangle down), Ac-Ry-NH_2 (open square), Ac-yR-NH_2 (open diamond), Ac-Yr-NH_2 (open triangle up), Ac-yr-NH_2 (open hexagon). D-amino acids are indicated by small letters and L –amino acids are indicated by capital letters (Gupta, et al., 2008).

4.1.4 MLE activity always correlates with P1 anchor preferences

Hydrogen-bond usage, stearic requirements and in particular the failure of dipeptides lacking the aromatic side chain further supported the assumption that the effect was mediated by dimorphic P1 pocket. The P1-pocket of HLA-DR1 (DRB*0101) contains β86 residue, which results in a preference for aromatic and to lower extent for aliphatic anchor residues (Rammensee, et al., 1997). This residue determines the depth of Pocket 1. P1 is deep if β86 residue is glycine (G) and is shallow if it is Valine (V) (Ong, et al., 1991). Aliphatic side chain anchors are preferred for HLA-DR1 molecule with β86V residue. This is due to better stearic accommodation of aromatic anchors at deep β86G pocket and aliphatic anchors for small β86V pocket. Detailed preferred anchors for various HLA-DR alleles having Gly/Val dimorphism at position β86 is mentioned in Table 1 (Rammensee, et al., 1997).

Table 1: Correlation of preferred anchors for Gly/Val dimorphism at position β86 residue of HLA-DR molecules.

Residue β86	HLA-DRB allele	Anchor residue ligand
Glycine	1*0101, 1*0302, 1*0401, 1*0701, 1*0801, 1*1101, 1*1302, 1*1402, 1*1502, 1*1601, 1*1602, 3*0101, 3*0202, 5*0101, 5*0102	F, Y, W (M, I, L, V)
Valine	1*0102, 1*0301, 1*0402, 1*0804, 1*1102, 1*1301, 1*1401, 1*1501, 1*1503, 3*0201, 3*0301, 4*0101, 4*0103, 5*0201, 5*0202	M, I, L, V

To validate and confirm whether these anchor preferences are also reflected in catalytic activity of dipeptide, a set of dipeptides were tested in which the tyrosine (Y) residue of Ac-YR-NH_2 was replaced by other aromatic anchors, phenylalanine (F), and tryptophan (W) or by the aliphatic amino acid anchors leucine (L), valine (V), isoleucine (I), and methionine (M). Glutamine (E) was used as negative control. Modified dipeptides were tested for antigen loading capacity. In line with expectation, strongest enhancement for antigen loading of HLA-DR1 (β86G) was noticed with dipeptides containing aromatic side chain anchors F,Y or W (Figure 5, left) and weaker activity was shown by dipeptide with aliphatic anchors L,M,I,V. Importantly Ac-ER-NH_2 did not show any enhancement, where E belongs to the residue not fitting in the P1 pocket of HLA-DR1 (Figure 5 left) (Fleckenstein, et al., 1996).

So far dipeptides showed promising results in antigen loading experiments. Next thing was to confirm if the dipeptides are also able to dissociate ligand from MHC class II proteins. To evaluate their influence on complex dissociation, soluble class II MHC molecules were preloaded with the CLIP peptide. CLIP peptide, to form stable HLA-DR1/CLIP complex. CLIP is derived from the invariant chain peptide and is known to bind, HLA-DR with lower affinity than HA306-318. The complex was incubated with free HA306-318 peptide, in presence and absence of peptide-MLE. Although the peptide binds to HLA-DR1 with

lower affinity than HA306-318 still only 10% of the HLA-DR:CLIP complex decayed spontaneously until 20h. However the effect was remarkably increased in presence of dipeptides. Maximum dissociation was obtained with Ac-FR-NH_2. Nearly 50% of the MHC molecules lost their CLIP after 5h when Ac-FR-NH_2 was present in the reaction. (Figure 5, right). The order of activity obtained was F>Y>L, and is in line to P1 anchor preference for HLA-DR1 molecules. So the dipeptides which can form complex efficiently are also able to dissociate the complex in similar manner, a phenomenon executed by HLA-DM at endosomal pH 5.0, for loading antigens on MHC proteins in human body.

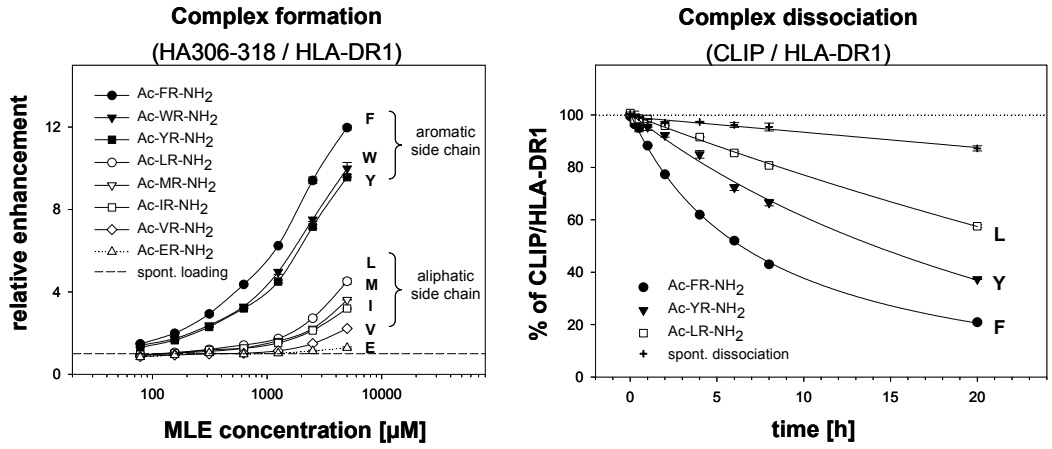

Figure 5: Impact of peptide-MLE on complex formation and dissociation. Left Panel:Complex formation. Structural requirements of the catalytic anchor side chain. Bulky hydrophobic side chains are preferred anchor for pocket 1 in HLA-DR1 protein. The relative enhancement with complex formation of HA306-318/HLA-DR1 is shown for the dipeptide derivatives Ac-FR-NH_2 (filled circle), Ac-WR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (open circle), Ac-MR-NH_2 (open triangle down), Ac-IR-NH_2 (open square), Ac-VR-NH_2 (open diamond), Ac-ER-NH_2 (open triangle up). The experiment was carried with titrated amounts of dipeptides. Right panel: Complex dissociation. A complex dissociation of CLIP/HLA-DR1 was determined in the presence of 10mM Ac-FR-NH_2 (filled circle), Ac-YR-NH_2 (filled triangle down), Ac-LR-NH_2 (open square) and in the absence of any dipeptide (cross). At indicated time points the percentage of remaining CLIP/HLA-DR1 complex was determined by ELISA. Complex dissociation was carried out in the presence of 200µg/ml free HA306-318 (Gupta, et al., 2008).

4.1.5 Peptide-MLE can trigger reversible ligand exchange

So far the catalytic effect of peptide-MLE was determined on loading of 'empty' MHC molecule. Execution of the dissociation experiment in the absence of free peptide indicated that it is in fact a reversible ligand-exchange is catalyzed. This is evident in the fact that in the absence of free HA306-318 peptide, no apparent spontaneous decay is observed, even if dipeptides are present in the assay (Figure 6a). However conditions looked different when during the assay excess of free HA306-318 peptide was added (Figure 6b). Under

these conditions in less than 2h, 50% of the HLA-DR1/CLIP complex was dissociated, in the presence of Ac-FR-NH_2. Notably in the absence of the peptide-MLE, 80% of the complex still remained intact after 64h incubation. This indicated that peptide-MLE are able to increase also the off rates of peptide-ligands. Also here anchor preference dictates the role of MLE in accelerating the reversible ligand exchange. In the presence of Ac-YR-NH_2 and Ac-LR-NH_2 during ligand exchange the half life of complex was reduced <4h and <10h. Control Ac-AR-NH_2 had a very limited effect (Figure 6b).

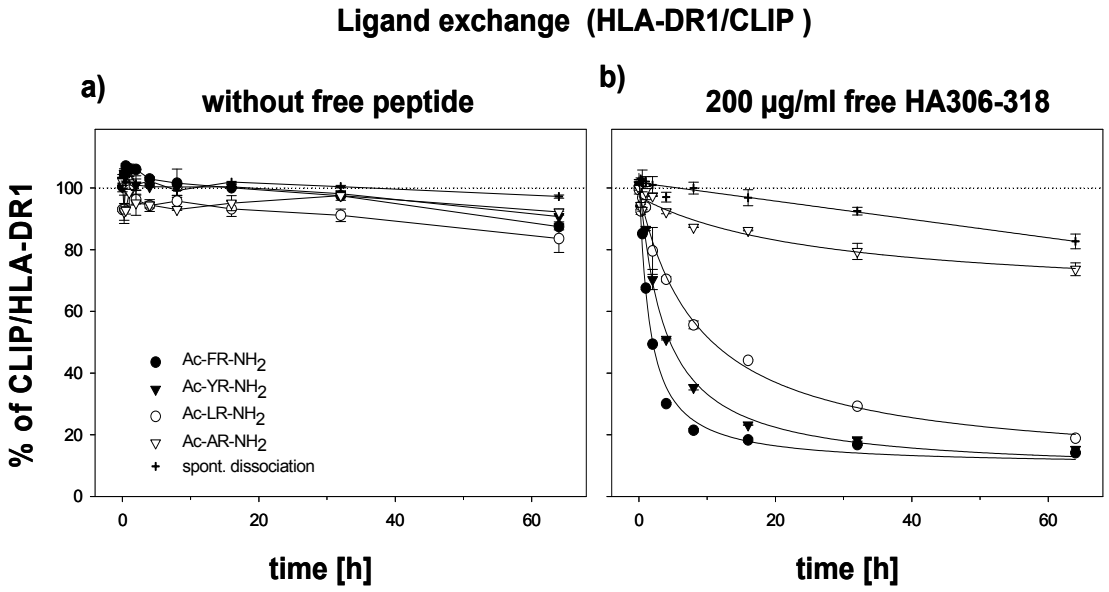

Figure 6: Dipeptides trigger reversible ligand exchange. To experimentally demonstrate the phenomenon that dipeptides can catalyze the reversible ligand exchange, complex dissociation of CLIP/ HLA-DR1 was carried out in presence of peptide MLE without addition of HA306-318 (figure left) or in the presence of 200µg/ml of free HA306-318 (figure right). The ligand experiment was carried out in presence of 10mM Ac-FR-NH_2, (filled circle), Ac-YR-NH_2 (filled triangle), Ac-LR-NH_2 (open circle), Ac-AR-NH_2 (open triangle) or no catalysts (cross) was used. At fixed time intervals samples were withdrawn in kinetic manner and percentage of CLIP/HLA-DR1 complex remaining was determined.

4.1.6 Pocket-1 of HLA-DR1 as target for peptide-MLE

Uptil now it has been shown that the activity of rationally designed dipeptides correlates with the side chain preference of pocket-1. To confirm and formally demonstrate that side chains of dipeptide-MLE actually interacts with pocket-1, mutants of HLA-DR1 were generated in which the glycine residue at β86 position of P1 was replaced either by valine (β86G→V) or by tyrosine (β86G→Y). β86V represents natural dimorphic alternate to β86G in HLA-DR.

The unnatural substitution β86Y, has been shown to block the pocket 1 by its tyrosine residue making MHC molecule permanently receptive with elevated on rate of antigen

loading (Natarajan, et al., 1999). Here only those peptides can bind whose binding does not depend on contribution from pocket-1.Therefore loading experiments were carried out with ABL908-922, a pentadecapeptide derived from the ABL kinase. Binding ability of ABL908-922 peptide was checked on HLA-DR1 variants by loading the antigen in the absence of MLE. The ABL 908-922 peptide was found to bind equally well to wt HLA-DR1 (β86G) and HLA-DR1 (β86G→V) with maximum value for loading reaching 60,000 cpm. A much higher peptide loading with elevated spontaneous on rate was observed with HLA-DR (β86G→Y), where maximum value reached to 180,000 cpm. This effect was due to inherent elevated self receptiveness (Figure 7 upper panel).

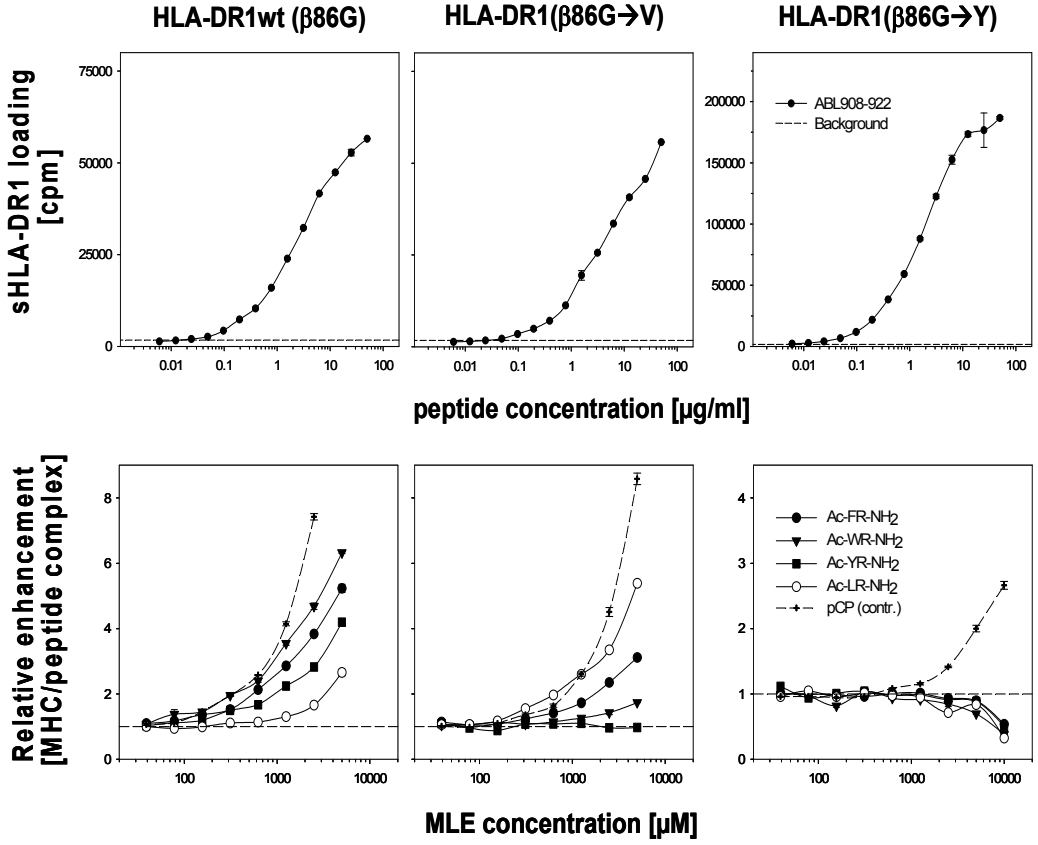

Figure 7: Catalytic dipeptides are allele selective and target pocket 1 of HLA-DR1 molecule. a) Binding of ABL908-922 peptide to HLA-DR1 and mutant proteins. In order to confirm the catalytic effect of dipeptide on HLA-DR1 wt and mutant proteins, binding of newly identified epitope ABL908-922 from ABL kinase was checked in an *in vitro* loading assay. Spontaneous loading of ABL908-922 is shown for wild type HLA-DR1 wt (β86G) (upper left figure) and for mutant HLA-DR1 (β86G→V) (upper middle figure) and HLA-DR1 (β86G→Y) (upper right figure). Amount of HLA-DR1 used was 150nM. b) Allele selective effect of peptide-MLE. ABL908-922 peptide that binds to wild type as well as mutated forms of HLA-DR1 as shown above was used for the experiment. For HLA-DR1 wt (β86G) and for HLA-DR1 (β86G→V) 1.5µg/ml and for HLA-DR1 (β86G→Y) 0.2 µg/ml of ABL908-922 peptide was used. Briefly 150nM of each HLA-DR1 wt (β86G) (lower left figure) and for mutant HLA-DR1 (β86G→V) (lower middle figure) and HLA-DR1 (β86G→Y) (lower right figure) was incubated with ABL908-922 peptide in presence and absence of MLE. The effect on HLA-DR1 loading is shown for Ac-FR-NH_2 (filled circle), Ac-WR-NH_2 (filled inverted triangle), Ac-YR-NH_2 (square) and Ac-LR-NH_2 open circle) and for p-chlorophenol (pCP; cross with dashed line), a

simple aromatic MLE compound acting independent of P1. Increment in complex formation is expressed as relative enhancement in reference to the spontaneous complex formation (without MLE). The formation of ABL 908-922/HLA-DR1 complex is expressed in counts per minute (cpm). Background signal is indicated by dashed line (Gupta, et al., 2008).

In order to confirm the putative role of P1, ABL908-922 peptide was incubated with HLA-DR variants with sets of dipeptides. As shown before in the case of HLA-DR1 wt (β86G) aromatic dipeptides showed stronger enhancements of ABL908-922 peptide. This was similar to the result obtained for the HA306-318 peptide, in which stronger enhancement was obtained with the dipeptides having side chain aromatic anchors like (F>,W>,Y) and only a weak catalytic effect was observed with Ac-LR-NH_2 with aliphatic anchor. However when experiments were carried out using HLA-DR1 Mut (β86G→V), the pattern was totally reversed. Best enhancement was obtained with Ac-LR-NH_2, and much weaker enhancement was observed by dipeptides with aromatic side chains. The result was in line to the reported anchor preference of shallow pocket 1. Notably, with HLA-DR (β86G→Y), none of the dipeptides showed any enhancement in antigen loading, at best slight reduction evident. However a chemical compound 4-chloro phenol (pCP), which works irrespective of pocket-1 (Hopner, et al., 2006; Marin-Esteban, et al., 2004), showed MLE effect even on the receptive HLA-DR (β86G→Y). This suggests that antigen loading on this receptive HLA-DR can also be enhanced. The same compound also showed enhancement on other two HLA-DR1 variants (β86G, and β86G→V) independent of pocket-1 (Figure 7 lower). Thus peptide-MLE show allele specific enhancement on HLA-DR1 protein, by targeting pocket 1.

4.1.7 Summary of catalytic activity of short peptides

In addition testing dipeptides some tripeptide and tetrapeptide sets, were also tested to see whether the extension of amino acids, could improve antigen loading capacity. Calculation of the mean catalytic activity of peptide-MLE was done by fitting single rectangular hyperbola curve and coefficient was calculated by forming average of the starting slope as shown in detail on figure 8 and then plotting the values to calculate loading rate enhancement (Table 2).

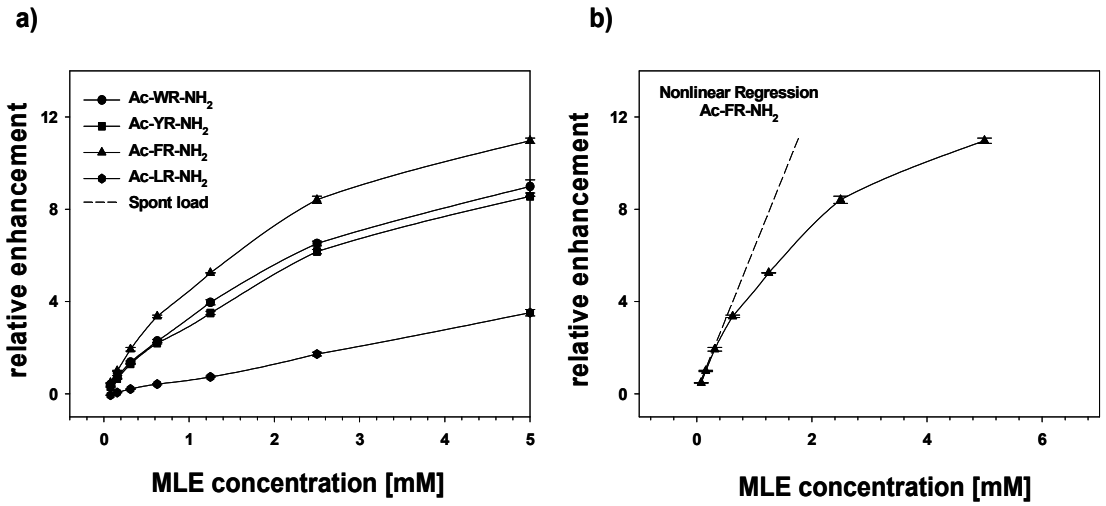

Figure 8: Calculation of peptide loading rate enhancement on HLA-DR1 : Catalytic rate enhancement was calculated by fitting single rectangular hyperbola with 2 parameters, and coefficient was calculated by forming average of the starting slope. Equation y=ax/(b+x) was utilized for calculating catalytic rate enhancement, where variables are x= MLE concentration, y= Relative enhancement, and parameters are a=maximum catatytic activity, b=c50.

Table 2: Calculation of peptide loading rate enhancement on HLA-DR1 (β86G)

Dipeptides	a	b mM	a/b [x 10^3 M-1]	Rel. activity (Ac-FR-NH_2) [%]
Ac-FR-NH_2	16.6	2.52	6.6	100
Ac-WR-NH_2	15.1	3.4	4.4	67
Ac-YR-NH_2	15.1	3.8	4.0	61
Ac-LR-NH_2	1.5	2.1	0.7	11

Similar to Table 2, mean catalytic activity of peptide-MLE for the loading of soluble HLA-DR1 with biotinylated HA306-318 was calculated for the various sets of dipeptides and is summarized in the (Table 3). As mentioned before maximum activity was observed with aromatic side chain anchors and minimum with aliphatic side chain anchors, on HLA-DR1 (β86G). Catalytic rate enhancement coefficient for short peptides was determined as mentioned, for Ac-FR-NH_2 6.5mM^{-1}, Ac-YR-NH_2 3.7mM^{-1}, Ac-WR-NH_2 3.5mM^{-1} .In reference to Ac-FR-NH_2 (activity considered 100%), dipeptides with aliphatic side chains showed activity between 12%(L) and 4%(V). Summarizing the data revealed that the core sequence for MLE effect seems to be 2 amino acids or dipeptides, as tri peptides did not show better effect, and their activity was lying close to dipeptides with aliphatic side chain anchors. Similar miserable activity was seen with peptides derived from invariant chain (LRMK, LRMKLPK) (Xu, et al., 2001). These so called 'Ii-key' peptides have been reported to facilitate antigen loading on MHC class II molecules by targeting site located outside P1-pocket, close to the invariant chain binding region termed as 'allosteric site'. The activity of these 'Ii-key' peptides however, was not better than our tripeptides. Activity of N-terminal fragment of the invariant chain octapeptide (LRKPPKPV), was also checked as this fragment was reported to exhibit antigen loading and catalyze the self

release of IC106-120 (CLIP) peptide (Kropshofer, et al., 1995). At least in our experimental system we could not observe any MLE effect.

Table 3: Catalytic activity of short peptide sets on the loading of soluble HLA-DR1 with HA 306-318

	Compound†	Catalytic Rate Enhancement†† [x 10^3 M^{-1}]	rel. cat. Activity††† [%]
a)	**Minimal peptide-MLE**		
1	Ac-FR-NH_2	6.5 +/- 1.2	100
2	Ac-YR-NH_2	3.7 +/- 1.0	57
3	Ac-WR-NH_2	3.5 +/- 0.9	54
4	Ac-LR-NH_2	0.76 +/- 0.08	12
5	Ac-MR-NH_2	0.52 +/- 0.00	8
6	Ac-IR-NH_2	0.43 +/- 0.01	7
7	Ac-VR-NH_2	0.25 +/- 0.02	4
8	Ac-ER-NH_2	0.02 +/- 0.02	0
9	Ac-AR-NH_2	0.00 +/- 0.00	0
b)	**Catalytic tripeptides**		
10	YFR	0.68 +/- 0.29	11
11	YKT	0.59 +/- 0.12	9
12	KYV	0.51 +/- 0.15	8
13	GYV	0.49 +/- 0.16	8
c)	**'Invariant Chain'-derived peptides**		
14	LRMKLPK	0.98 +/- 0.21	15
15	LRMK	0.53 +/- 0.15	8
16	LRKPPKPV	0.00 +/- 0.00	0

† 'Minimal peptide-MLE' and 'catalytic tripeptides' are introduced in this study, catalytic activity for 'invariant chain derived peptides' has been reported for LRMK and LRMKLPK (Xu, et al., 2001) and for LRKPPKPV (Kropshofer, et al., 1995).
††The 'Catalytic Rate Enhancement' coefficient (k) represents the relative increase of the spontaneous loading rate (r_{spont}) in the presence of the catalytic peptide (P_{cat}). The total rate (r_{tot}) can be calculated by ($r_{tot} = r_{spont}+k\ [P_{cat}]\ r_{spont}$).
† † † ''rel. cat. Activity'' indicates the relative catalytic activity of peptide derivatives and is expressed as percentage in reference to the catalytic rate enhancement of Ac-FR-NH_2.(Gupta, et al., 2008).

4.1.8 Peptide-MLE can enhance antigen loading on living antigen presenting cell (APC)

It is known from our previous works, that simple synthetic chemical compounds can enhance the antigen loading directly on the cell surface expressing MHC molecules (Hopner, et al., 2006; Marin-Esteban, et al., 2004). To validate that this applies also to dipeptides, antigen loading on cell surface MHC molecules were determined on antigen presenting cells. Cell surface loading was determined by confocal microscopy and flow cytometry (FACS).

For the confocal experiments, 722.221 cells expressing HLA-DR1 GFP fusion protein (GFP linked to βchain 722.221-DRb1GFP) were used, as green fluorescent protein can be easily detected by imaging. 722.221-DRb1GFP cells were incubated with biotinylated

HA306-318 peptide in absence and presence of Ac-FR-NH_2. After the incubation, cells were washed and stained with fluorescence labeled streptavidin (SA-Cy5). Subsequently imaging of the cells was done by confocal laser scanning microscopy. The expression of the HLA-DR on the cell surface can be seen by green fluorescence and peptide by red fluorescence. Ac-FR-NH_2 showed a striking enhancement in the amount of peptide binding (red colour) at cell surface MHC molecules, which colocalized nicely with the green fluorescence expressed by MHC molecules. However the cells which were incubated with HA306-318 peptide in the absence of Ac-FR-NH_2 did not show any detectable peptide loading at cell surface. Independently co-localization of the signal is also evident from the line plot in which, green line signifies the HLA-DR expression and the red line suggests the peptide signal (Figure 8).

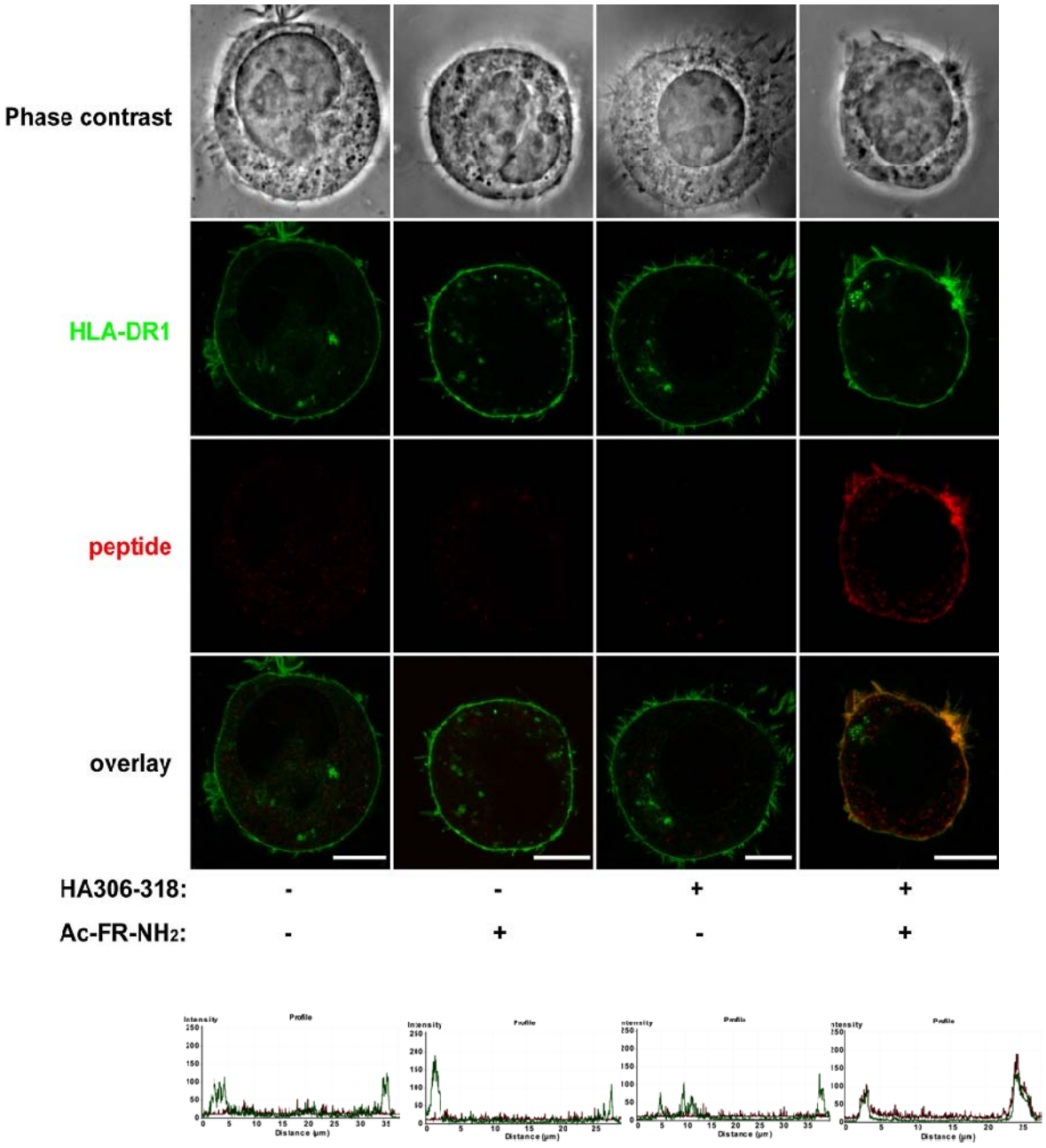

Figure 8: Enhanced loading of cell surface MHC determined by confocal laser scanning microscopy. 721.221-DRb1GFP cells expressing a GFP-tagged HLA-DR1 molecule were used as APC and were incubated with 20µg/ml biotinylated HA306-318 peptide in the absence or presence of 2.5mM of Ac-FR-NH_2.After staining with streptavidin-Cy5 images were taken by confocal laser scanning microscopy. Scale bar represents 10µm. Lower panel denotes the line scan across the cell. Colocalization of HLA-DR1-GFP with HA306-318 peptide (red) (Gupta, et al., 2008).

Confocal microscopy showed that the peptide-MLE can enhance the antigen loading on the cell surface MHC molecules expressed on APC. To confirm that the MLE catalyzed antigen loading is only affected on cells expressing MHC, FACS analysis was carried out. L929 mouse fibroblast lacking MHC class II were transfected with human HLA-DR1 to express MHC at cell surface. Cells were incubated with biotinylated ABL908-922 peptide,

then staining and FACS analysis was performed . Only fibroblasts expressing HLA-DR1 show antigen loading, whereas cells lacking MHC class II (cells-MHC) do not show any loading (Figure 9a). In experiment with other cell type 722.221, expressing HLA-DR1 were incubated with biotin labeled HA306-318 peptide in presence of Ac-FR-NH_2. Without MLE presence geomean of antigen loading enhancement is 11.8. However in presence of Ac-FR-NH_2 the values reach 194.8. Ac-FR-NH_2 could enhance the antigen load by nearly 17 times, evident by shift shown by red curve (Figure 9b).

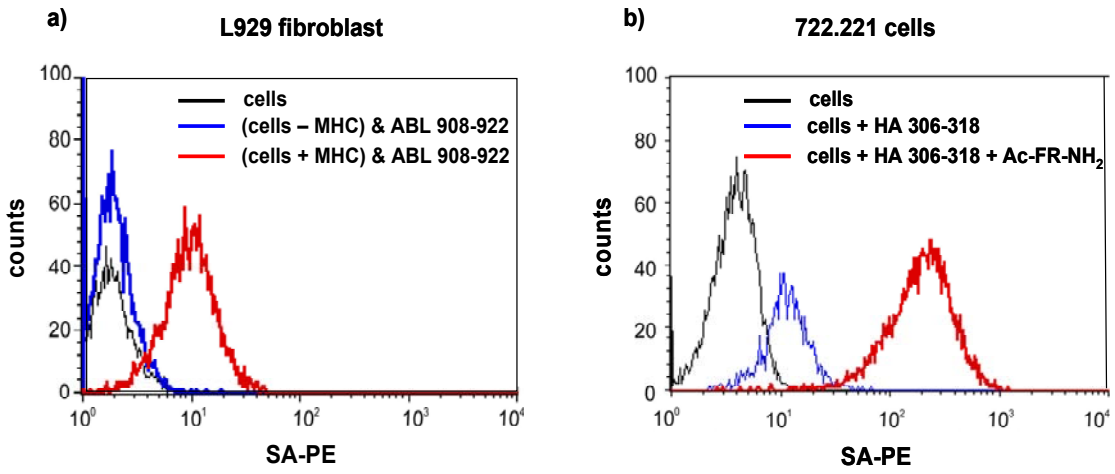

Figure 9: Cell surface antigen loading determined by flow cytometry. A) 50µg/ml of ABL908-922 peptide was incubated with L929 fibroblast transfected by HLA-DR1 shown by red curve, blue curve shows loading of L929 fibroblast without MHC. And black curve denotes the background for cell alone. B) 1µg/ml of biotinylated HA306-318 peptide was incubated with 722.221 cells in absence of MLE shown by blue curve and in presence of 5mM Ac-FR-NH_2 shown by red curve, black curve denotes the background for cell alone. The staining of cells was done by streptavidin phycoerythrin conjugate (SA-PE).

To further quantitate the loading enhancement in detail flow cytometric analysis was performed. In this experiment fibroblast cells were used that do not express any endogenous or self MHC class II molecules, but they were transfected with full length versions of wt HLA-DR1 (β86G), or mutated versions HLA-DR1 Mut (β86G→V), and HLA-DR1 mutant (β86G→Y). From the soluble HLA-DR protein data we knew that ABL908-922 binds to all the three HLA-DR variants. The binding of this epitope was nearly similar in HLA-DR1 (β86G), HLA-DR1 Mut (β86G→V) with geomean values 5 for β86G and 6 for β86G→V, HLA-DR, (Figure 10 upper left and upper middle) however high spontaneous on rate was observed with the HLA-DR1 Mut (β86G→Y) expressing fibroblasts, as evident from geomean 11 (Figure 10 upper right). As internal control no binding was observed in L929 fibroblast without any MHC class II molecule.

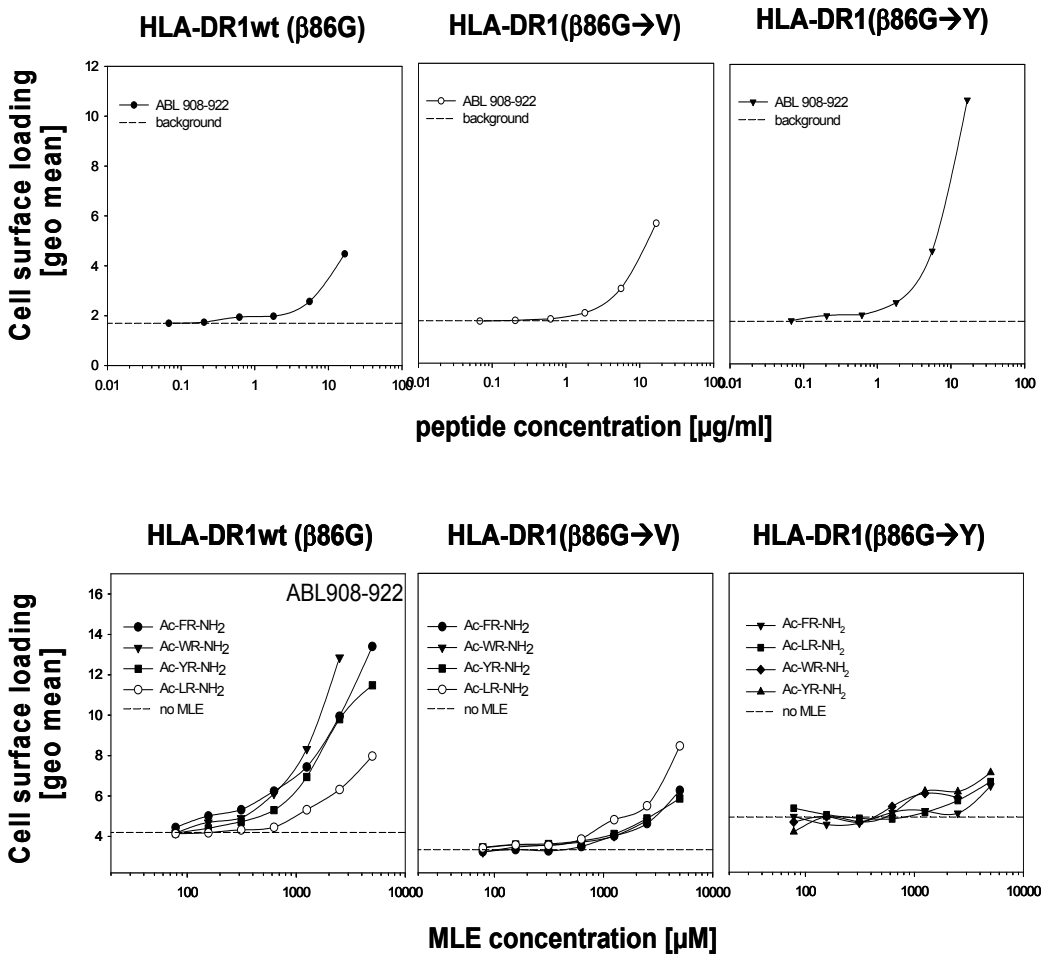

Figure 10: Dipeptide can mediate loading of ABL908-922 peptide on cell surface MHC molecule in allele selective manner determined by FACS. a) Binding of ABL908-922 peptide on cell surface MHC molecules. L929 mouse fibroblast transfectants expressing either wt HLA-DR1 (upper left) or mutated HLA-DR1 (β86G→V) (upper middle) or HLA-DR1 (β86G→Y) (upper right) were incubated with titrated amount of ABL908-922 peptide to confirm the binding of peptide on cell surface MHC. b) Catalytic effect of dipeptide on cell surface MHC molecule expressing either HLA-DR1 wt (lower left) or mutant HLA-DR1 (β86G→V) (lower middle) or HLA-DR1 (β86G→Y) (lower right) molecule were incubated with ABL908-922 peptide in presence and absence of dipeptides. Fibroblast cells were incubated with 12µg/ml of biotinylated ABL908-922 peptide (for HLA-DR1 wild type and mutant HLA-DR1 (β86G→V)) or 9µg/ml for HLA-DR1 (β86G→Y) mutant. The result is shown as Ac-FR-NH_2 (filled circle), Ac-WR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (open circle), for lower left and middle figure, and for lower right figure Ac-FR-NH_2 (filled triangle down), Ac-LR-NH_2 (filled square), Ac-WR-NH_2 (filled diamond), Ac-YR-NH_2 (filled triangle). Cell surface antigen loading was analyzed by FACS. Cell surface MHC loading is expressed as geometrical mean (geomean) (Gupta, et al., 2008).

For MLE experiments, cells were incubated with biotinylated ABL908-922 epitope, in presence and absence of titrated amount of catalytic dipeptide. The cell surface MHC loading results in presence of peptide-MLE showed similar trend as obtained with the soluble protein. On the fibroblast expressing wt HLA-DR1 (β86G), strongest enhancement was observed with aromatic peptide-MLE (F,W,Y), while the aliphatic Ac-LR-NH_2 showed the weakest effect (Figure 10 lower panel). The panel was reversed with HLA-DR1 (β86G→V), in which the best catalytic effect was observed with Ac-LR-NH_2 and weaker effect were obtained with aromatic peptide MLE (F,W,Y). However no enhancement was seen on HLA-DR1 mutant (β86G→Y) (Figure 10 lower panel).

To confirm the result with another peptide, we used IC106-120, peptide. The binding of IC 106-120 was nearly similar in HLA-DR1 (β86G), HLA-DR1 Mut (β86G→V) (Figure 11 upper left and upper middle) high spontaneous on rate was observed with the HLA-DR1 Mut (β86G→Y) expressing fibroblasts, (Figure 11 upper right). Cell surface loading was performed with biotinylated IC106-120 peptide in presence and absence of peptide-MLE. Aromatic MLE showed better enhancement on HLA-DR1 (β86G) while the MLE with aliphatic side chain showed best enhancement on HLA-DR1 (β86G→V) HLA-DR1 mutant (β86G→Y) loading could not be enhanced by peptide-MLE (Figure 11 lower). Hence cell surface antigen loading enhancement correlated directly with allele specific anchor preferences for P1 pocket for respective HLA-DR1 alleles.

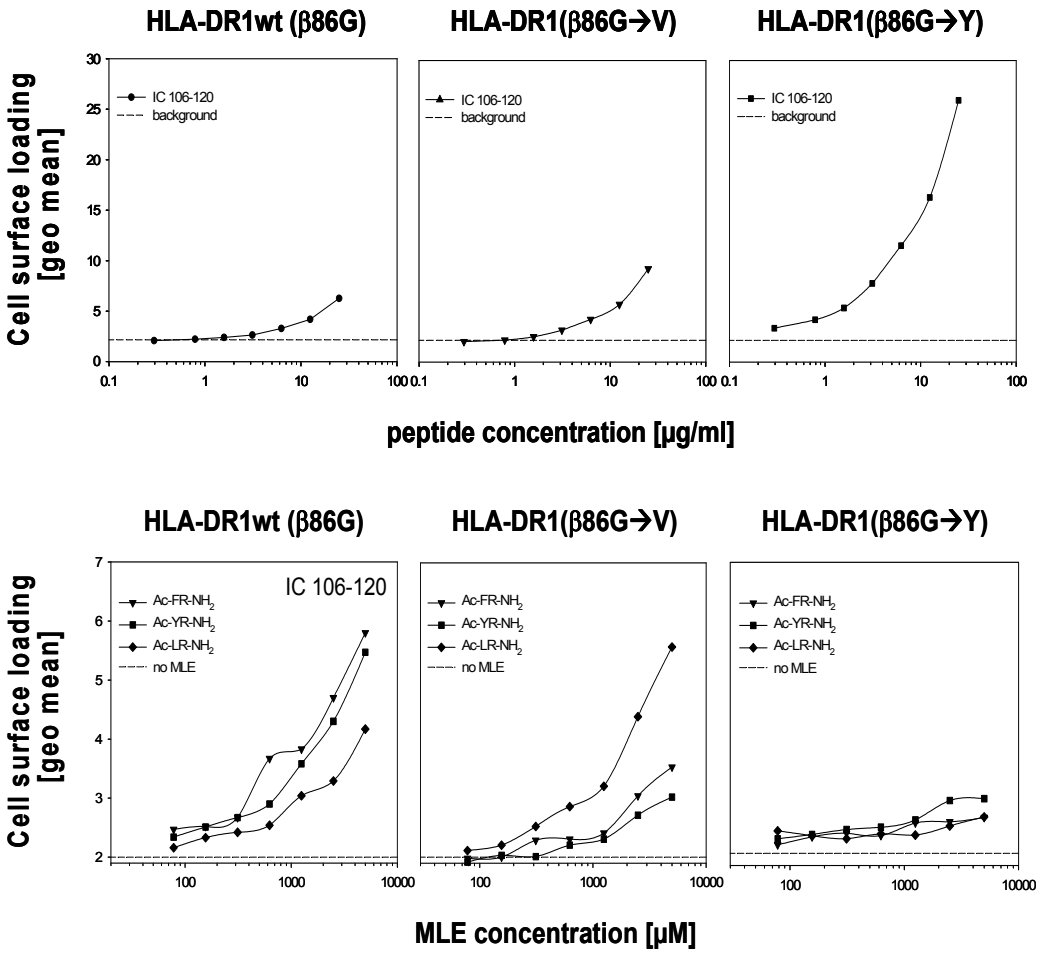

Figure 11: Dipeptide can mediate loading of CLIP (IC 106-120) peptide on cell surface MHC molecule in allele selective manner determined by FACS. a) Binding of IC106-120 peptide on cell surface MHC molecules. L929 mouse fibroblast transfectants expressing either wt HLA-DR1 (upper left) or mutated HLA-DR1 (β86G→V) (upper middle) or HLA-DR1 (β86G→Y) (upper right) were incubated with titrated amount of IC106-120 peptide to confirm the binding of peptide on cell surface MHC. b) Catalytic effect of dipeptide on cell surface MHC molecule expressing either HLA-DR1 wt (lower left) or mutant HLA-DR1 (β86G→V) (lower middle) or HLA-DR1 (β86G→Y) (lower right) molecule were incubated with IC106-120 peptide in presence and absence of dipeptides. Fibroblast cells were incubated with 7µg/ml of biotinylated IC106-120 peptide for HLA-DR1 wild type, 3µg/ml for mutant HLA-DR1 (β86G→V)) or 0.3µg/ml for HLA-DR1 (β86G→Y) mutant. The results are shown as Ac-FR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (filled diamond). Cell surface antigen loading was analyzed by FACS. Cell surface MHC loading is expressed as geometrical mean (geomean).

4.1.9 Enhancement of antigen loading on dendritic cells

Dipeptides have already shown enhancement of peptide loading on HLA-DR1 expressing Epstein Barr Virus (EBV) transformed B cells and also on fibroblasts expressing HLA-DR. To confirm if dipeptides also show similar MLE effect on dendritic cells (DC), as the protease repertoire present in dendritic cells is different from other cell types, and these cells are most important cells during immune responses. Bone marrow derived dendritic cells (BM-DC) were made from HLA-DR1-transgenic mice, expressing wt HLA-DR1 (β86G). The immature dendritic cells obtained were matured by addition of lipopolysaccharide (LPS). Mouse bone marrow progenitor cells were CD11c+ and MHC Class II+, as confirmed by flow cytometry. The maturation status of dendritic cells were confirmed by staining for HLA-DR expression and CD86, a activation marker for DC. Matured dendritic cells expressed high levels of HLA-DR and CD86 (Figure 12 left). For performing APC loading matured BM-DC were loaded with biotinylated-HA306-318 peptide, in presence and absence of 3mM of peptide-MLE (Figure 12 right). Enhancement obtained was similar to obtained before with other cell types. Ac-FR-NH2 showed best enhancement and Ac-LR-NH2 showed weakest enhancement, in antigen loading. Thus MLE works not only on cell lines but the effect can also be seen on delicate dendritic cells.

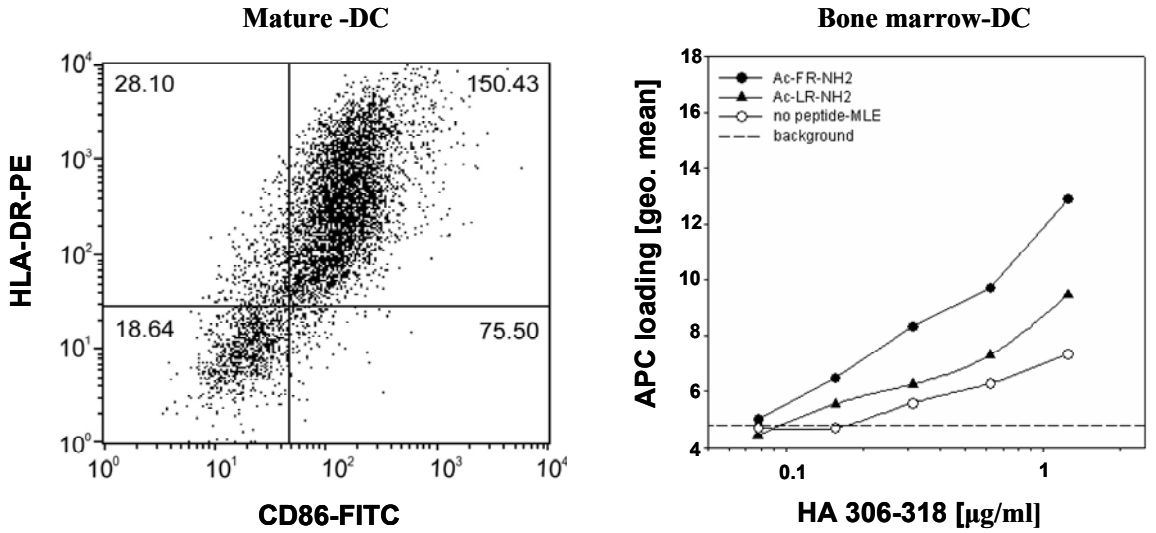

Figure 12: Impact of dipeptides in accelerating antigen loading on mouse bone marrow derived dendritic cells. left figure Maturation status of bone marrow derived dendritic cells (BM-DC). Maturation status was confirmed by staining for HLA-DR and and CD86, a activation marker for DC: To formally confirm the effect of dipeptides on bone marrow derived dendritic cells originating from HLA-DR1 transgenic mice. Right figure The cells were incubated with titrated amounts of biotinylated HA306-318 peptide in the absence or presence of 3mM peptide MLE. Surface-loading was determined after 4h by FACS analysis. The result is shown in form of dose response curve mentioned as Ac-FR-NH_2, (filled circle), Ac-LR-NH_2 (filled triangle), no peptide-MLE (open circle), background (dashed line). Cell surface MHC loading is expressed as geometrical mean (geomean).

4.1.10 Amplification of the antigen specific CD4+ T cell response *in vitro*

Since the dipeptides enhance the antigen loading on cell surface MHC molecules, the increased loading efficiency of antigen should directly be translated into an amplification of the CD4+ T cell response. Amplification of antigen specific immune response was confirmed by mouse hybridomas and a human CD4+T cell line.

The experiments were carried out with SaABL/G2 hybridoma. This hybridoma is known to recognize the ABL908-922 antigen on both wt HLA-DR1 (β86G), or on mutant HLA-DR1 (β86G→V). L929 fibroblasts expressing either wt HLA-DR1 (β86G), or mutated version HLA-DR1 mut (β86G→V), were incubated with sub-threshold concentration of ABL908-922 epitope, in presence of titrated amount of peptide-MLE (Figure 13 upper) with titrated amount of ABL 908-922 epitope in presence of fixed amount of peptide MLE (Figure 13 lower). After washing, the antigen loaded APC were presented to SaABL/G2 hybridoma. Strongest antigen specific response was observed when the APC were loaded in the presence of the dipeptides. The trend of antigen specific enhancement shown by the peptide-MLE directly correlated with previous results of antigen loading on cell surface MHC molecules, and moreover the effect directly correlated with the allele specific preference for pocket-1.Thus in line to previous results aromatic dipeptides (F,W,Y) showed maximum enhancement of CD4+ T cell response on HLA-DR1 (β86G) (Figure 13 upper left), while Ac-LR-NH_2, showed best stimulation on cells expressing HLA-DR1 mut (β86G→V) (Figure 13 upper right), having shallow P1-pocket. Thus enhance antigen loaded by dipeptide is directly reflected in amplification of immune responses in allele selective manner. As shown in the lower panel presence of fixed concentration of peptide-MLE, at titrated ABL908-922 concentration shifted the dose response curve towards lower antigen concentration. Aromatic dipeptides (F,W,Y) showed maximum enhancement of CD4+ T cell response on HLA-DR1 (β86G**)** (Figure 13 lower left), while Ac-LR-NH_2, showed best stimulation on cells expressing HLA-DR1 mut (β86G→V), having shallow P1-pocket (Figure 13 lower right).

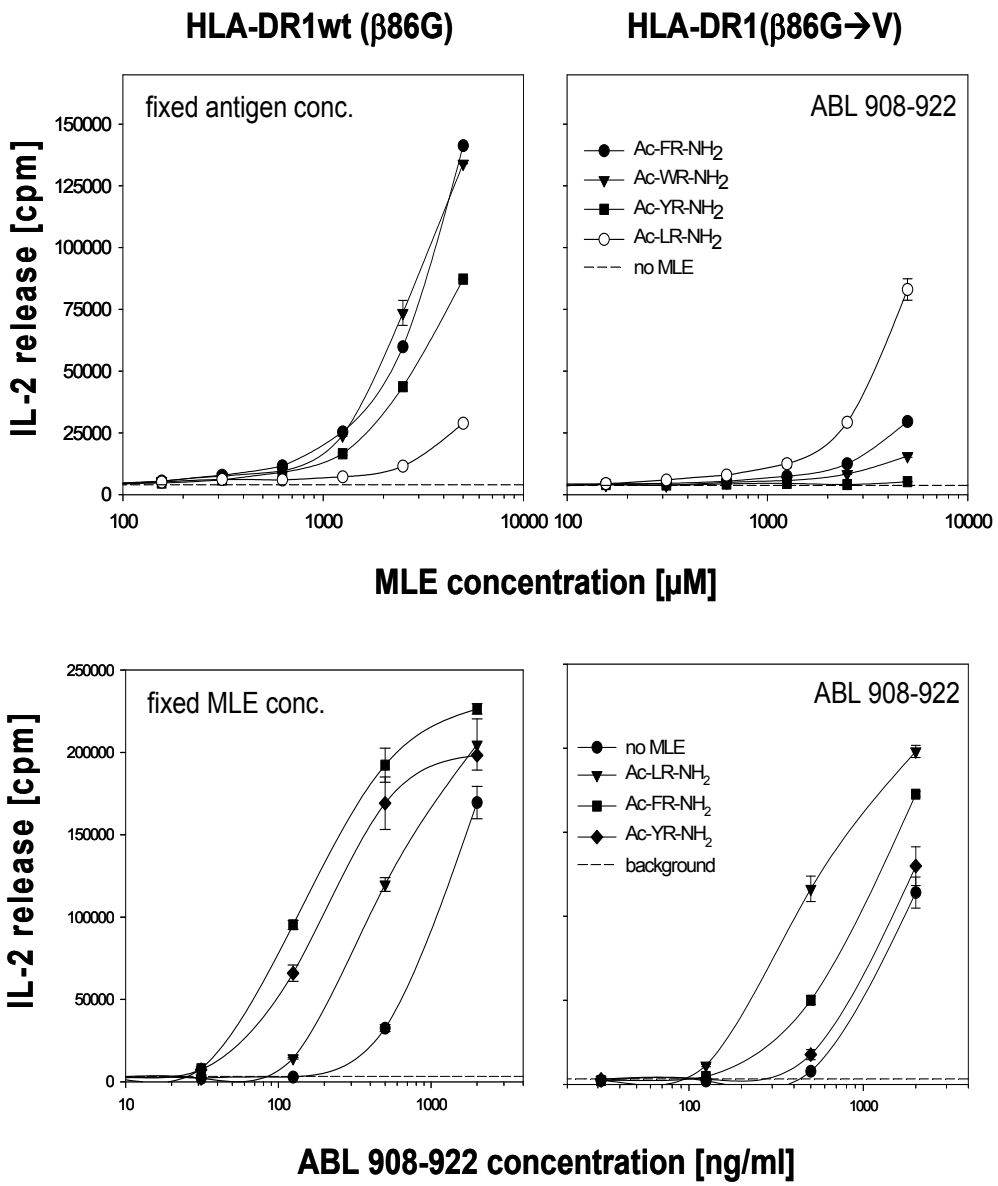

Figure 13: Impact of peptide MLE in amplification of ABL 908-922 specific CD4+ T cell response in allele specific manner. L929 mouse fibroblast transfectants expressing either wt HLA-DR1 (upper left) or mutated HLA-DR1 (β86G→V) (upper right) were incubated with 150ng/ml or 300ng/ ml of ABL908-922 peptide in presence or absence of titrated amount of peptide-MLE Ac-FR-NH_2 (filled circle), Ac-WR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (open circle) for 4h (figure upper panel) , or titration of ABL908-922 peptide in presence and absence of 5mM fixed concentration of peptide-MLE, shown as Ac-LR-NH_2 (filled inverted triangle) Ac-FR-NH_2 (filled square), Ac-YR-NH_2 (filled diamond) or no MLE (filled circle) was performed on the fibroblasts (figure lower panel), subsequently the APC were washed and used to challenge SaABL1/G2, an ABL908-922 antigen specific T cell hybridoma.The amplification of the T cell response in presence of either titrated amount of MLE (upper panel) (Gupta, et al., 2008) or fixed amount of MLE (lower panel) is expressed as IL-2 release. Dashed line represent the T cell response triggered in the absence of any peptide-MLE (upper figure), and T and APC background (lower figure).

The effect of peptide-MLE on the CD4+ T cell response was further validated in an *in vitro* T cell assays with mouse T cell hybridoma, EvHA/X5 which are HLA-DR1 restricted and specific for HA306-318 antigen. HLA-DR1 expressing EBV cells 722.221 were incubated with sub threshold antigen concentration and titrated amount of peptide-MLE (Figure 14 left) or a fixed MLE and titrated antigen concentration (Figure 14 right). Presence of peptide-MLE enhanced the HA specific response to maximum extent in the concentration range of 2-3mM. Ac-FR-NH_2 showed best enhancement (Figure 14 left). As shown in the right panel presence of fixed concentration of peptide-MLE, shifted the dose response curve of the antigen upto 50 folds toward lower antigen concentration (Figure 14 right)**.** While the half-maximal response in the absence of peptide-MLE was detected at 31ng/ml, for EvHA/X5 however presence of Ac-FR-NH_2 lowered the threshold to 0.65ng/ml, and weaker effect was shown by Ac-LR-NH_2. Thus dipeptides are able to shift the dose response to lowest threshold.

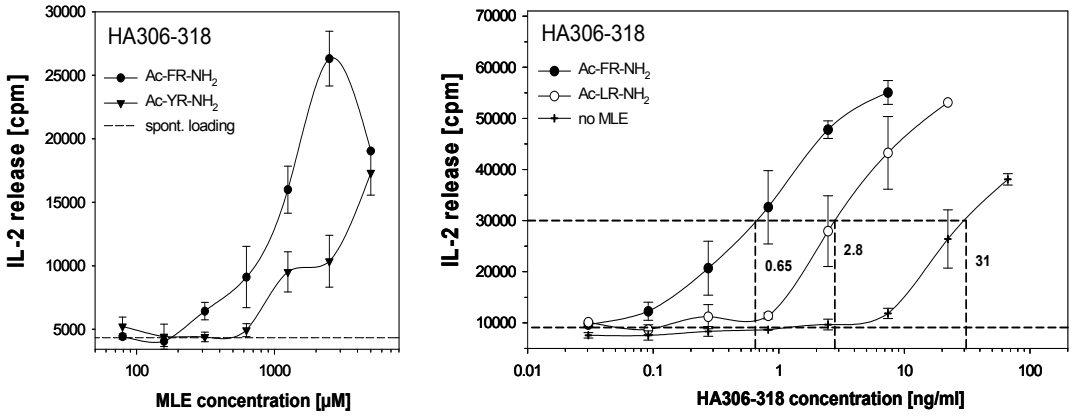

Figure 14: Impact of peptide MLE in amplification of HA306-318 specific CD4+ T cell response. To demonstrate that peptide-MLE can amplify HA306-318 antigen specific CD4+ T cell response, EBV transformed B lymphoblastoid cell lines 722.221, expressing HLA-DR1 were used as APC and were incubated with 15ng/ml of HA306-318, in presence and absence of titrated amount of peptide-MLE (figure left), or 722.221 cells were incubated with titrated amount of HA306-318, in presence of fixed 5mM peptide-MLE concentration for 48h.Subsequently the APC were presented to HLA-DR1 restricted/HA specific mouse T cell hybridoma EvHA/X5 respectively, without any pulse wash.The dose response curves are shown as Ac-FR-NH_2 (filled circle), Ac-YR-NH_2 (filled inverted triangle), Ac-LR-NH_2 (open circle) or no MLE (cross), Dashed line represent the T cell response triggered in the absence of any peptide-MLE (figure left), and T and APC background (figure right).The amplification of the T cell response in presence of either titrated amount of MLE (figure left) or fixed amount of MLE (figure right) is expressed as IL-2 release (Gupta, et al., 2008).

To confirm the effect of peptide-MLE on human CD4+ T cells, PD2 cell line was used. This CD4+ T cell line is HLA-DR1 restricted and HA306-318 antigen specific. HLA-DR1

expressing APC (HTR) were incubated with sub threshold antigen (HA306-318) concentration and titrated amount of peptide-MLE (Figure 15 left) or fixed MLE and titrated antigen concentration (Figure 15 right) in presence of PD2 T cell line. Enhancement observed was in following sequence Ac-FR-NH_2>Ac-YR-NH_2>Ac-LR-NH_2 (Figure 15 left). The amplification obtained at fixed concentration of Ac-FR-NH_2 was similar to obtained with EvHA/X5, with shift of dose response of HA to nearly 50 fold, towards lower antigen concentration. The half-maximal response in the absence of peptide-MLE was detected at 14ng/ml, for PD2, the presence of Ac-FR-NH_2 lowered the threshold to 0.23ng/ml (Figure 15 right). Enhancement observed was in following sequence Ac-FR-NH_2>Ac-YR-NH_2>Ac-LR-NH_2. Thus peptide-MLE also function when applied on human cell system.

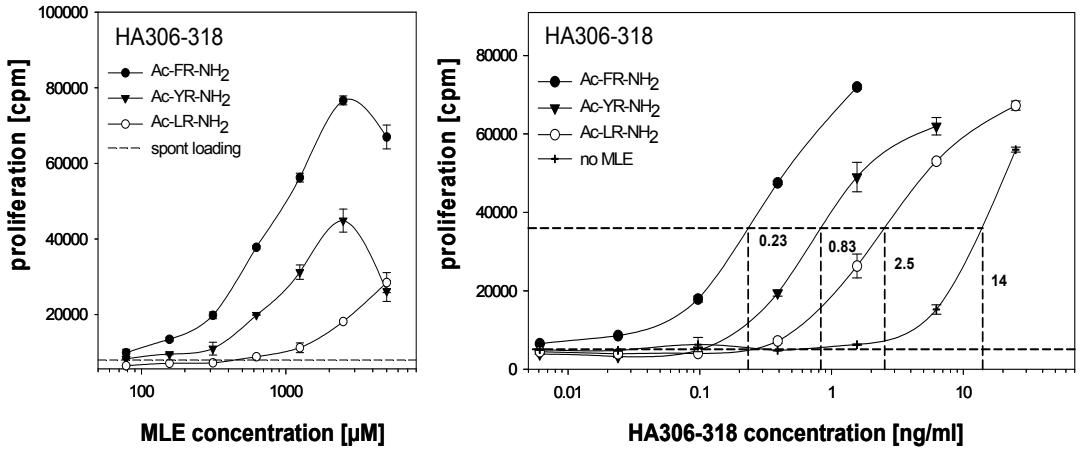

Figure 15: Amplification of HA306-318 specific human CD4+ T cell response *in vitro*. To formally demonstrate that peptide-MLE can amplify HA306-318 antigen specific human CD4+ T cell response, HTR cells were used as APC and were incubated with 2ng/ml of HA306-318, in presence and absence of titrated amount of peptide-MLE (figure left), or HTR cells were incubated with titrated amount of HA306-318, in presence of fixed 5mM of peptide-MLE, Ac-FR-NH_2, Ac-LR-NH_2 or 3mM of Ac-YR-NH_2 for 48h. Subsequently the APC were presented to HLA-DR1 restricted/HA specific human CD4+ T cell line respectively, without any pulse wash. The dose response curves are shown as Ac-FR-NH_2 (filled circle), Ac-YR-NH_2 (filled inverted triangle), Ac-LR-NH_2 (open circle) or no MLE (cross), Dashed line represent the T cell response triggered in the absence of any peptide-MLE (figure left), and T and APC background (figure right).The amplification of the T cell response in presence of either titrated amount of MLE (figure left) or fixed amount of MLE (figure right) is expressed as IL-2 release (Gupta, et al., 2008).

4.1.11 Amplification of the antigen specific CD4+ T cell response *ex vivo*

Finally in order to confirm if our immune modulator peptide-MLE can amplify the antigen specific CD4+ T cell response, also with '*natural*' primary cells, HLA-DR1-tg mice was immunized with HA306-318 antigen or with NY-ESO-1 89-101, a CD4+ T cell epitope derived from NY-ESO-1 protein associated with various solid tumors (Chen, et al., 2004). After 12 days lymph node cells were isolated from the mice and were rechallenged with 5ng/ml of HA306-318 (Figure 16 right) or 50ng/ml of NY-ESO-1 89-101 (Figure 16 left) in presence or absence of 2.5mM Ac-FR-NH_2. Antigen specific *ex vivo* immune response was determined using IFN-γ ELISPOT. In this assay each dot represents IFN-γ secreting cells, and amount of IFN-γ secretion was enhanced when Ac-FR-NH_2 was present in the reaction.
In line to our previous observations, Ac-FR-NH_2 increased significantly the sensitivity of the assay. Thus the short peptides showing MLE activity are able to amplify immune responses also in primary cultures containing 'natural' CD4+ T cells and professional APC.

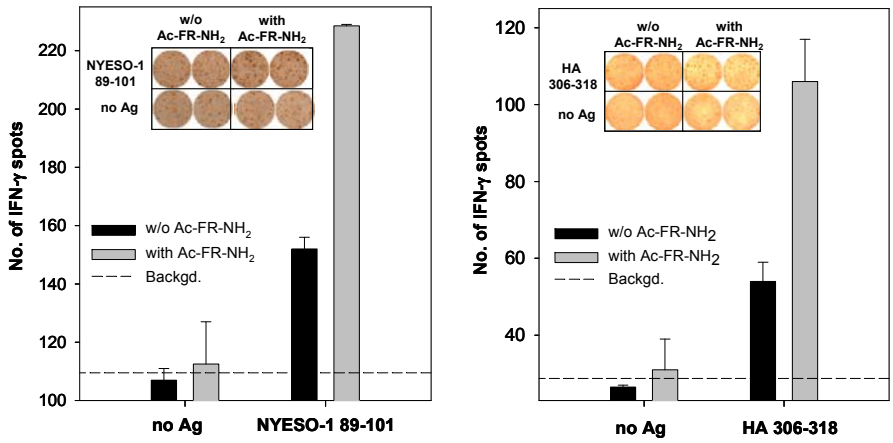

Figure 16: Impact of peptide MLE in amplification of *ex vivo* antigen specific CD4+ T cell response. To confirm, if dipeptides are able to amplify *ex vivo* antigen specific response, HLA-DR1tg mice were primed with 5µg HA306-318 or 10 µg NY-ESO-1 89-101. Lymph node cells were isolated and challenged by either 5ng/ml of HA306-318 (figure right) or 50ng/ml of NY-ESO-1 89-101(figure left) respectively , in absence and presence of 2.5mM of Ac-FR-NH_2, to monitor the *ex vivo* T cell response. Picture of individual spot in response to HA306-318 (right figure) or NY-ESO-189-101 (left figure) is shown as obtained from ELISPOT plate capture. Each spot originates from a single IFN-γ secreting cell. Dashed line indicates background signal. Bars represent the number of spots detected in absence (black bar) or presence of Ac-FR-NH_2 (grey bar). *Ex vivo* T cell response was determined by ELISPOT (Gupta, et al., 2008).

To summarize this section, the data suggest amino acid side chains of short peptides can trigger the reversible ligand-exchange, by targeting polymorphic P1 pocket of MHC class II molecule. The catalytic activity of dipeptides such as Tyr-Arg (YR) was stereo-specific and could be enhanced by modifications addressing the conserved H-bond network near the P1 pocket of the MHC molecule. This could enhance both antigen-loading and ligand-release and strictly correlated with reported anchor preferences of P1, the specific target site for the catalytic side chain of the dipeptide. The effect was evident also in CD4+ T cell assays, where the allele-selective influence of the dipeptides translated into increased sensitivities of the antigen-specific immune response. Peptide-MLE could shift the antigen dose response curve upto 50 fold towards lower antigen concentration.

4.2 Characterization of molecular mechanism behind MLE mediated ligand exchange

MHC class II molecule conformations are dynamic. More importantly at least two critical MHC conformers exist, a long lived "non-receptive" form with closed MHC binding site and short lived "receptive" form with open binding site (Natarajan, et al., 1999; Rabinowitz, et al., 1998). Conformational transitions have also been postulated to be major reason behind MLE activity. So far MLE induced conformational shift is suggested and not proven. In this section experimental evidence for the conformational change is provided.

4.2.1 Spectral analysis

4.2.1.1 Monitoring of conformational shift by binding of ANS dye

8-Anilino-1-napthalenesulphonic acid (ANS) is known to bind to the hydrophobic patches of proteins causing ANS to emit fluorescence of higher magnitude and towards shorter wavelength. (Stryer, 1965). This blue shift can be used to probe changes in protein conformation, caused due to exposure of previously buried or hydrophobic/non-polar areas. Provided such that the formation of receptive state in the presence of MLE compounds is linked with conformational changes in MHC and ANS fluorescence should allow to detect MLE induced conformation transitions. In order to test our assumptions soluble HLA-DR1 wt (β86G) and mutant HLA-DR molecules with β86G→V and tyrosine mutants or receptive HLA-DR β86G→Y protein were incubated with 1-adamantane ethanol (1-AdEtOH) (a chemical MLE, known to target pocket 1, for MLE effect) and ANS dye. All the experiment were done in PBS, pH 7.4. Subsequently the mixture was scanned for emission wavelength in a luminescence spectrometer. Controls, PBS and HLA-DR alone did not show any auto fluorescence (data not shown). In the presence of HLA-DR1, the ANS fluorescence was slightly blue shifted. However the effect was significantly increased in the presence of 1-AdEtOH.
The HLA-DR wt which were incubated with 1-adamantane ethanol, showed marked enhancement in fluorescence intensity with subsequent shift of maximum emission wavelength (lambda max) towards blue region. The enhancement in the intensity are shown by red curve corresponding to 1-AdEtOH mediated conformational change in HLA-DR1. This HLA-DR allele is susceptible to 1-adamantane ethanol. Hence, this shift can be directly correlated to conformational shift involved in MLE mediated antigen loading. Solvent control with DMSO showed only a slight shift of HLA-DR1/ANS fluorescence. Importantly in control sets with HLA-DR1 mutant β86G→V or β86G→Y, no

enhancement in fluorescence intensity was observed (Figure 1, upper panel). These mutant alleles are non susceptible to 1-AdEtOH. The effect is particularly evident after substraction of DMSO/ANS fluorescence values from the fluorescence produced by 1-AdEtOH/ANS. The substracted values were plotted as shown in (Figure 1, lower panel). MLE causes a significant shift in MHC conformation in susceptible allele (HLA-DR1 β86G) but not on non-susceptible alleles. This conformational shift by the MLE is correlated with the induction of the 'receptive state'.

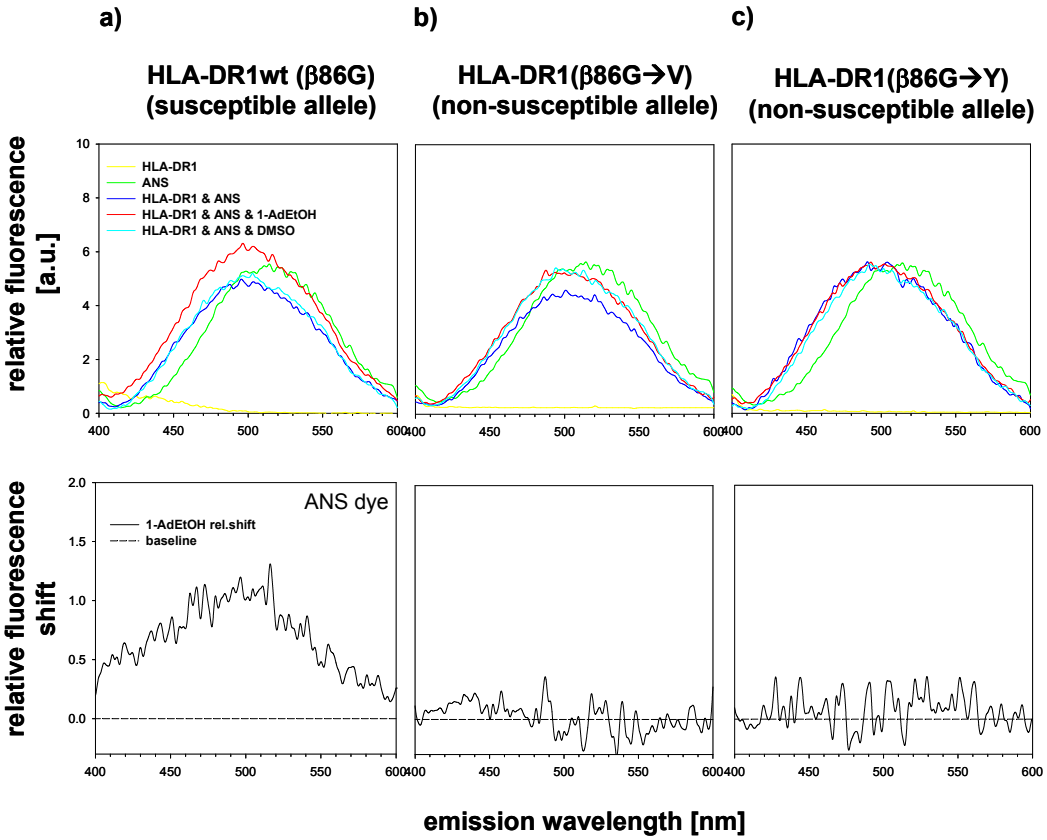

Figure 1: Impact of MLE on conformational shift monitored by ANS dye (upper panel). Recombinant soluble HLA-DR1 molecules were mutated inside P1 pocket and were used for the experiment. 1µM of HLA-DR1 wild type (β86G) (figure left) HLA-DR1 mutant β86G→V (middle figure) HLA-DR1 mutant β86G→Y (figure right) were incubated in presence of 100µM ANS dye in absence and presence of 1 mM of 1-AdEtOH. Subsequently emission wavelength scan was performed in luminescence spectrometer to determine enhancement in fluorescence intensity induced by MLE. Enhancement in fluorescence intensity is shown above in curve plot HLA-DR1 and 1-AdEtOH (red curve), 1% DMSO solvent control for 1-AdEtOH (cyan), HLA-DR1 alone (yellow), ANS (green) and HLA-DR1 and ANS (blue). Fluorescence intensity is shown as arbitrary units on Y-axis and wavelength scan is shown on X-axis. **Relative fluorescence in HLA-DR1 induced by MLE (lower panel).** Real or relative shift induced by 1-AdEtOH on HLA-DR1 wt (β86G) (figure left) HLA-DR1 mutant β86G→V (middle figure) HLA-DR1 mutant β86G→Y (figure right) were calculated by subtraction of fluorescence shift produced by DMSO (solvent control for 1-AdEtOH) from 1-AdEtOH values. MLE induced relative shift is shown by black zig-zag line in a wavelength emission scan. Dotted line shows the zero base line and Y-axis denoted the relative shift values and X-axis shows the wavelength scan.

4.2.1.2 Monitoring of conformational shift by intrinsic tryptophan fluorescence

Three aromatic amino acid residues (tryptophan, tyrosine and phenylalanine) present in proteins might contribute to their intrinsic fluorescence. After excitation most of the emissions are due to excitation of tryptophan residues with very few emissions caused due to phenylalanine and tyrosine residues. Previously the interaction between HLA-DR and HLA-DM has already been studied by measuring intrinsic tryptophan fluorescence (Chou and Sadegh-Nasseri, 2000). The HLA-DR molecule has 9 tryptophan residues, the residues located on the α-chains are 43, 121, 168, and 178 and residues on β-chain are 9, 61, 131, 153 and 188 but only α-43 and β-9, 61 and 153 residues belong to peptide binding site, residues α-43 and β-153 are located just near the pocket 1 of HLA-DR molecules (Stern, et al., 1994). Therefore we assumed that MLE might also affect the intrinsic fluorescence of tryptophan residues present in HLA-DR molecules, and better shifts might be observed with residues located close to peptide binding site. As a result of this conformational change some changes in the intrinsic tryptophan values, might be observed.

To validate our assumption the possibility of change in intrinsic tryptophan fluorescence in presence of MLE was examined. For this experiment soluble HLA-DR1 wt (β86G) and mutant proteins (HLA-DR1 β86G→V, HLA-DR1 β86G→Y).were incubated with 1-AdEtOH/PBS at pH 7.4. For measuring intrinsic tryptophan fluorescence the MLE treated HLA-DR samples were excited with 290nm (to avoid interference with tyrosine fluorescence) and emission spectra scan was obtained from 310nm to 410nm. DR samples alone showed some tryptophan fluorescence. HLA-DR1 wt (β86G) samples which were pre- incubated in presence of 1-AdEtOH showed enhanced intensity maxima of the emission fluorescence spectra as compared to the solvent DMSO alone (Figure 2, upper panel). However the samples with HLA-DR1 mutant (HLA-DR1 β86G→V and HLA-DR1 β86G→Y) proteins, no enhancement in tryptophan fluorescence intensity was observed. The intensity maxima produced by solvent DMSO control was nearly similar to 1-AdEtOH (no effective shift in case of the mutant proteins). Real enhancement of intrinsic tryptophan fluorescence was calculated by substracting DMSO control fluorescence values from 1-AdEtOH intensity values. Susceptible HLA-DR (β86G) wt showed shift in intrinsic tryptophan fluorescence during presence of 1-AdEtOH. No enhancement was observed in mutant HLA-DR molecules (Figure 2, lower panel).

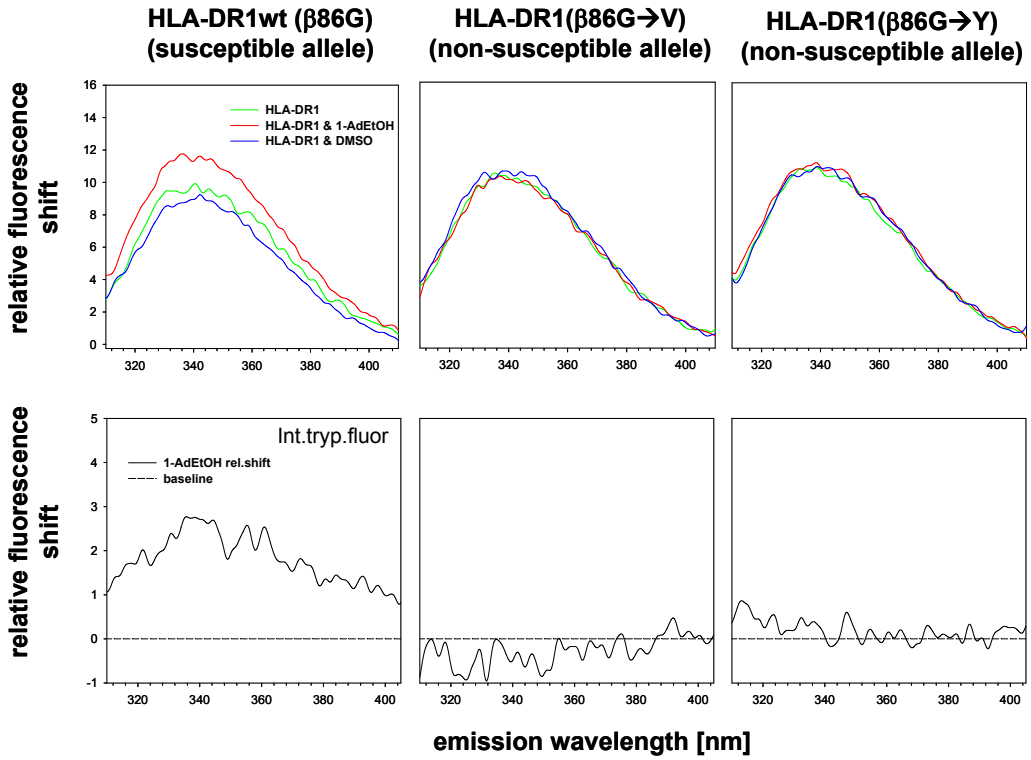

Figure 2: Impact of MLE on intrinsic tryptophan fluorescence of HLA-DR1 protein (upper panel). 800nM of recombinant soluble HLA-DR1 wild type (β86G) (figure left) HLA-DR1 mutant β86G→V (middle figure) HLA-DR1 mutant β86G→Y (figure right) were incubated in presence of 1mM of 1-AdEtOH. Subsequent emission wavelength scan was performed from 310-410nm after excitation at 290nm in a luminescence spectrometer for the determination of MLE induced shift in intrinsic tryptophan fluorescence. Enhancement in fluorescence intensity is shown above in curve plot HLA-DR1 and 1-AdEtOH (red curve), 1% DMSO control (blue), HLA-DR1 alone (green). Fluorescence intensity is shown as arbitrary units on Y-axis and wavelength scan is shown on X-axis. **Relative fluorescence in HLA-DR1 induced by MLE (lower panel).** Relative shift induced by 1-AdEtOH on HLA-DR1 wt (β86G) (figure left) HLA-DR1 mutant β86G→V (middle figure) HLA-DR1 mutant β86G→Y (figure right) were calculated by subtraction of fluorescence shift produced by DMSO from 1-AdEtOH values. MLE induced relative shift is shown by black zig-zag line in a wavelength emission scan. Dotted line shows the zero base line and Y-axis denoted the relative shift values and X-axis shows the wavelength scan.

Thus with ANS and intrinsic tryptophan fluorescence it could be shown that the conformational change could be correlated by occupation of P1 pocket by MLE compound.

4.2.2 Detection of receptive state with conformational specific antibodies

4.2.2.1 Conformational specific antibodies targeting the peptide binding site

Series of monoclonal antibodies (MEM 264, MEM 265, MEM 266, MEM 267,) recognizing the β subunit of HLA-DR1 were characterized (Carven, et al., 2004). These MEM antibodies have been reported to be specific for the empty conformation of HLA-DR1 as they react with the empty form and show no reactivity to the peptide loaded form (Carven GJ, JBC 2004). MEM-267, 264 and 265 recognize HLA-DR-β(50-69 region) whereas MEM-266 binds to C- terminal of DRβ (170-190 region) (Carven, et al., 2004) (Figure3).

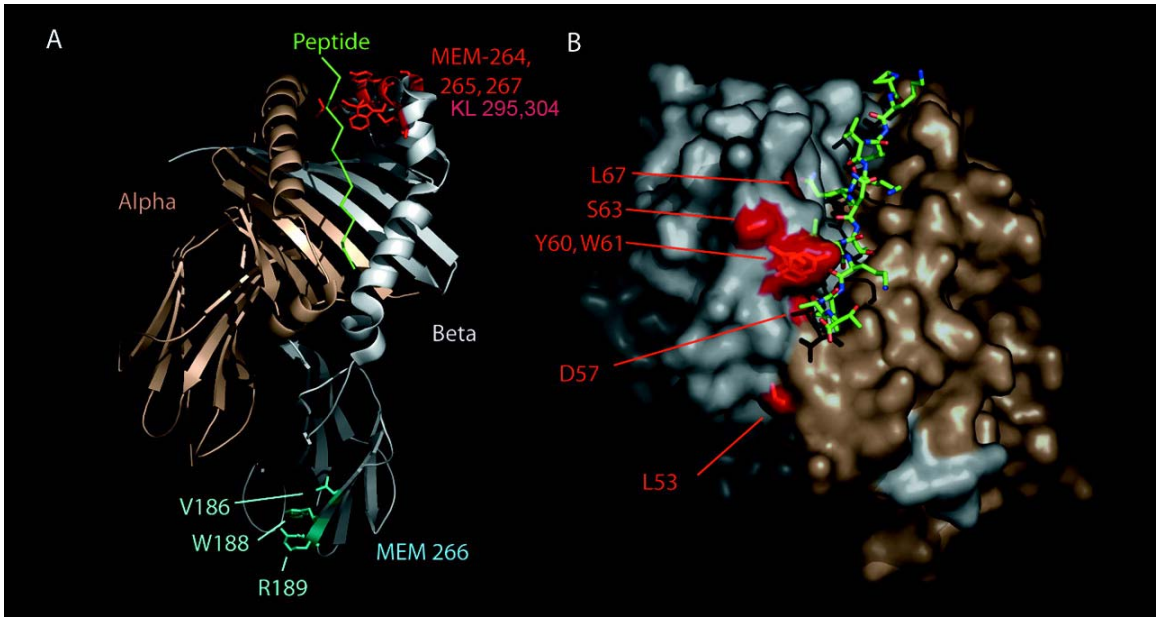

Figure 3: Epitope map of conformational specific antibodies. A. Diagrammatic representation of HLA-DR1 bound to HA-peptide. Residues for binding of conformational specific antibodies MEM 264,265,266,267 and KL-295 and 304 are shown in red color. Residues that bind to MEM-264, 265, 266, 267 are shown in cyan color. B. Surface view of these conformational sensitive MEM antibodies (Carven, et al., 2004).

MLE compounds enhance the antigen loading on MHC class II proteins by the inducing receptive state. We were interested in the behaviour of MEM-267 antibody. Biotin labelled soluble HLA-DR1 protein (produced in SF9 insect cells) was incubated in presence of 1-AdaEtOH or pCP or peptide-MLE (Ac-YR-NH_2) and the resulting reaction was captured by conformational sensitive MEM antibody or L243 (control) coated plates. The amount of HLA-DR binding was detected by ELISA. In line to publication the signal was reduced in the presence of HA306-318 peptide. Importantly the signal dramatically increased when MLE compounds were added. This was true for 1-AdEtOH (Figure 4a), pCP (Figure 4c), and importantly same effect was also seen with Ac-YR-NH_2 (Figure 4b). The enhance MEM reactivity obtained was not an signal artefact as signal stayed low in presence of

solvent control. The result obtained with Ac-YR-NH_2 was in contrast to previous publication, in which binding of dipeptide reduced the MEM reactivity (Carven, et al., 2004). The enhanced reactivity effect to MLE treated HLA-DR is only seen with conformation sensitive antibody however the effect was not seen with L243 antibody which binds equally to loaded and unloaded HLA-DR. The MEM reactivity experiments pointed to the detection of receptive state induced in MHC class II proteins in the presence of MLE.

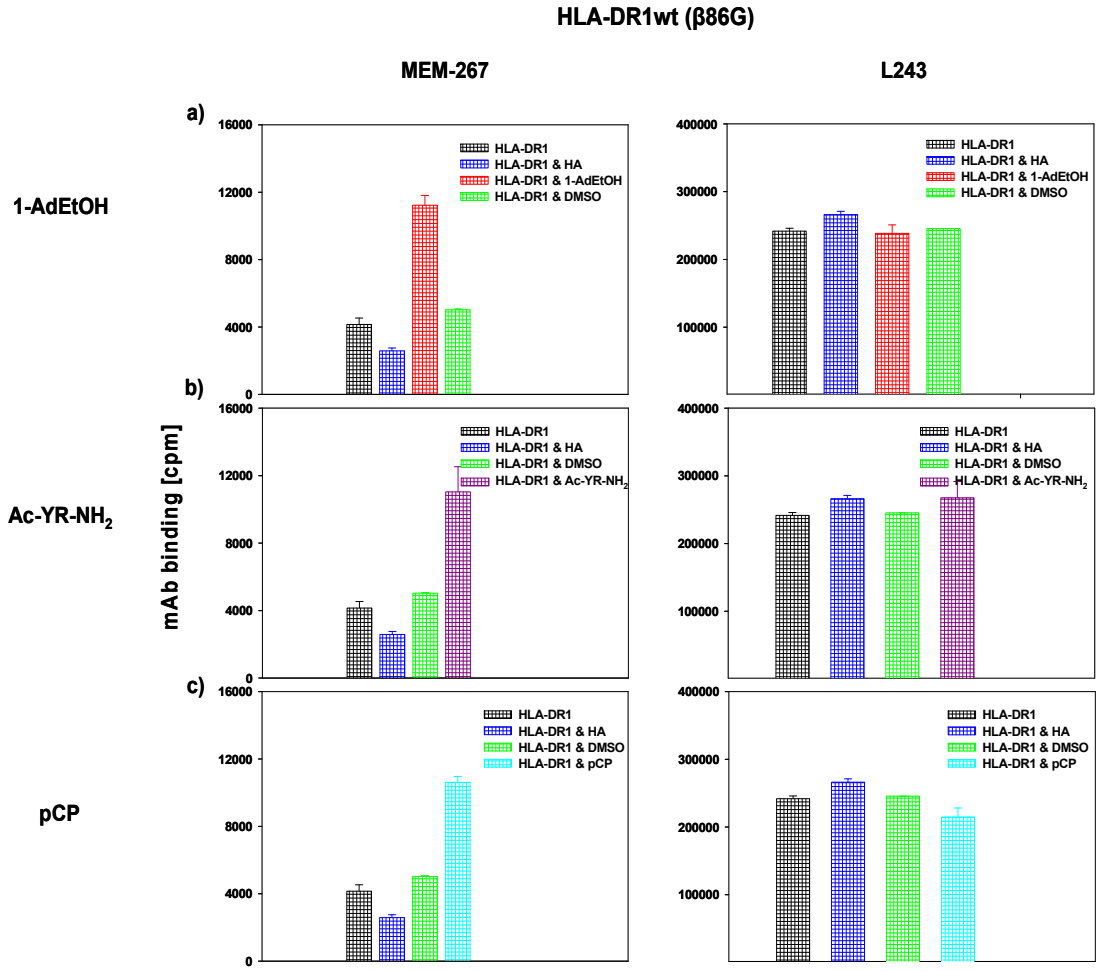

Figure 4: Impact of MLE on HLA-DR1 conformation shift detected by MEM antibodies. To determine the MEM reactivity to MLE treated HLA-DR1 wt soluble protein, experiment was setup with 500nM of biotin labelled HLA-DR1 in presence of MLE. Result is shown as bar chart. HLA-DR1 protein (black) incubated with 100µg/ml of HA306-318 peptide (blue), 1mM of 1-AdEtOH (red), solvent DMSO control for 1-AdEtOH (green), 10mM Ac-YR-NH_2 (purple), 10mM pCP (cyan). MEM reactivity is show in the left panel and the corresponding reactivity is shown in the right panel. Results are expressed as counts per minute.

The effect was also confirmed with other conformational specific antibodies KL-295 recognizing HLA-DR1 β (58-69) residue (LaPan, et al., 1992; Sato, et al., 2000), KL-304 β (57-68) residue (LaPan, et al., 1992; Santambrogio, et al., 1999). Experimental condition was same as mentioned above. Same phenomenon of enhanced antibody reactivity was

observed in presence of 1-AdEtOH (Figure 5 left). Above effect was not shown by L243 antibody, which served as an experimental control (Figure 5 right).

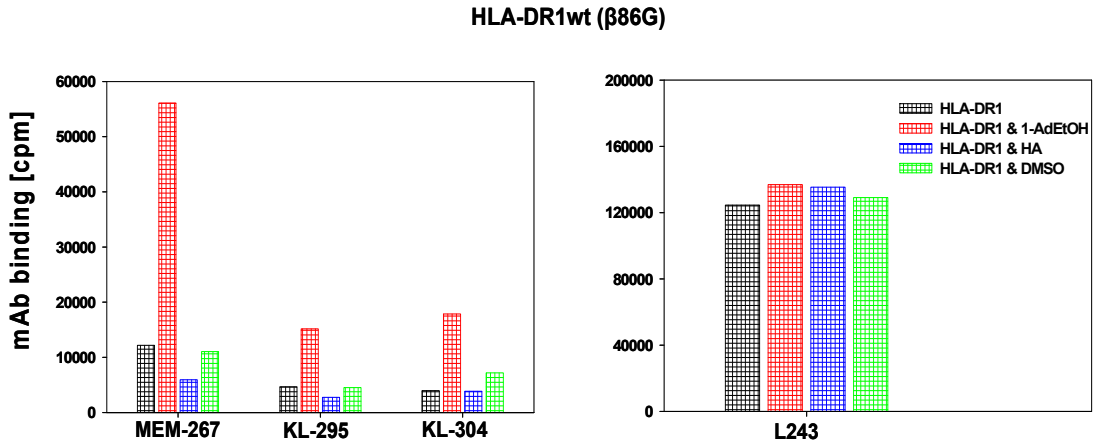

Figure 5: Impact of MLE on HLA-DR1 conformation detected by other conformational sensitive antibodies. The phenomenon of enhanced monoclonal antibody reactivity to MLE treated HLA-DR1 can also be observed with other conformational sensitive antibodies like KL-295, KL-304 in comparison to MEM-267 **(**figure left) and control L243 (figure right). The result is summarized in bar chart as HLA-DR1 (black), HLA-DR1 and 1-AdEtOH (red), HLA-DR1 and HA (blue), HLA-DR1 and 1% DMSO-solvent control for 1-AdEtOH (green). Enhanced monoclonal antibody binding was expressed as counts per minute on Y-axis.

To further justify our observation that MEM antibodies are probe for receptive MHC, we tried to observe the reactivity of these antibodies towards *'receptive'* HLA-DR molecules, in which β86G residue was mutated to tyrosine (Y) residue. This mutation blocks the pocket and makes MHC permanently receptive (Natarajan, et al., 1999). High MEM reactivity was observed with receptive HLA-DR1 molecule (β86G→Y) with counts reaching to 22,158 in comparison to HLA-DR1 wt β86G with 1,730 counts. This was in contrast to the previous publications where it has been shown that receptive HLA-DR is not showing any reactivity towards MEM antibodies (Carven, et al., 2004). Though the protocol used by them was different. However in my experiment the *'receptive'* HLA-DR molecule showed high MEM reactivity (Figure 6a). As from the previous data it is known that MLE could not enhance antigen loading on receptive HLA-DR1 molecule (Gupta, et al., 2008), similar to that no enhancement in MEM reactivity was observed with MLE, 1-AdEtOH, Ac-YR-NH_2, pCP, however with Ac-YR-NH_2 a significant reduction was observed, might be due to blockade of binding site. L243 served as a control (Figure 6b, c).

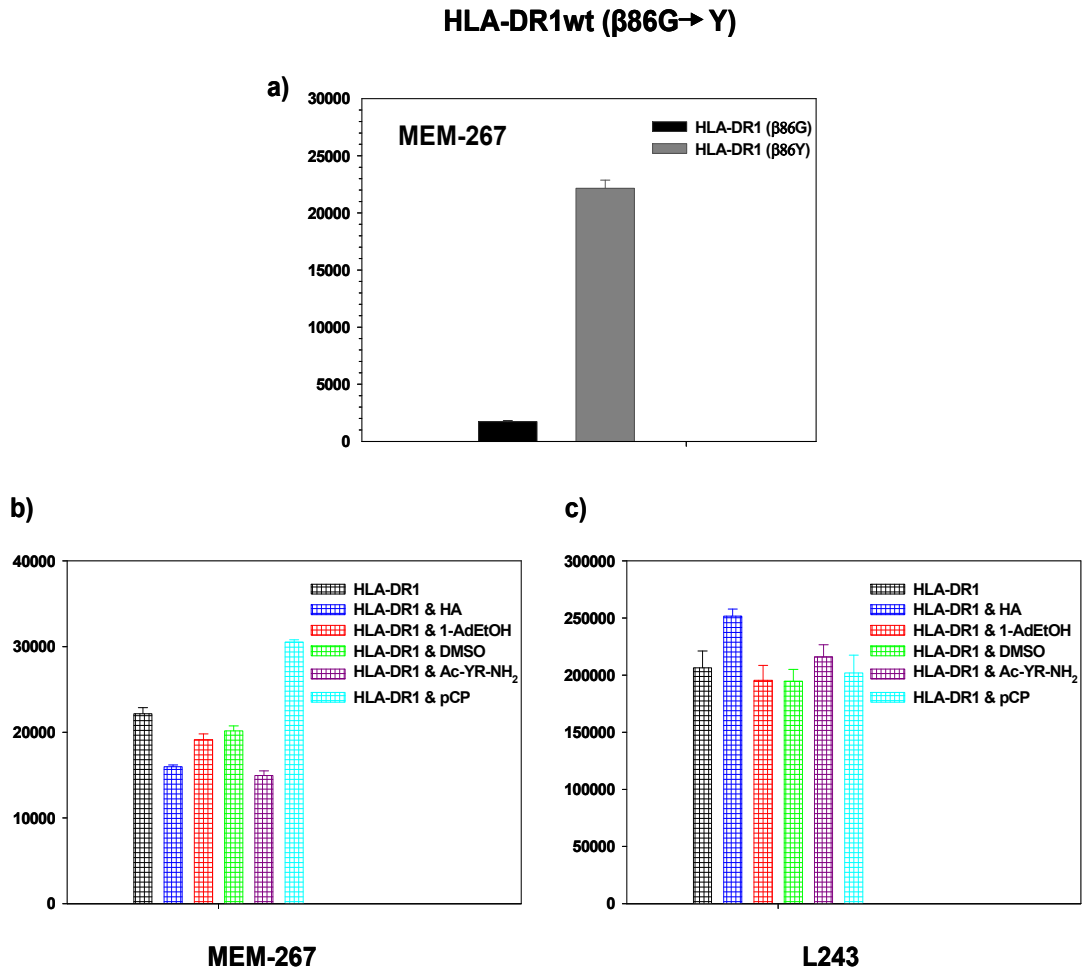

Figure 6: A. MEM reactivity towards receptive HLA-DR1 (β86G→Y) molecule. a) To confirm that MEM antibodies probe receptiveness in HLA-DR1 molecule experiments were carried out with biotin labelled recombinant HLA-DR1 mutant (β86G→Y) molecule, in comparison to wt HLA-DR molecule. The results are shown as vertical bar chart showing β86G (black), β86G→Y (grey). **b,c). Impact of MLE on receptive HLA-DR1 molecule.** Biotin labeled receptive HLA-DR1 protein was incubated with HA306-318 peptide, The result obtained is summarized as HLA-DR1 β86G→Y (black), HLA-DR1 and HA (blue), HLA-DR1 and 1-AdEtOH (red), HLA-DR1 and 1% DMSO-solvent control for 1- AdEtOH (green), HLA-DR1 and Ac-YR-NH_2 (purple), HLA-DR1 and pCP (cyan). Enhanced monoclonal antibody binding are expressed as counts per minute on Y-axis.

4.2.2.2 Conformational specific antibodies targeting site distant from peptide binding site

As the epitope of MEM 267 is in the MHC binding site, therefore we were interested in looking whether these conformational transitions are transported to COOH- terminus of the HLA-DR1 extracellular domain, which includes the last 5 residues in the β strand at the bottom of immunoglobulin like domain. Residues near to this region are known to interact with HLA-DM (Doebele, et al., 2000). Above experiment was repeated with MEM 266 antibody in comparison to MEM 267 antibody. Enhancement in the MEM reactivity was

observed with 1-AdEtOH, also with Ac-YR-NH_2. Thus these MEM 266 antibodies also show similar enhancement in reactivity in the presence of peptide MLE as well as chemical MLE (Figure 7).

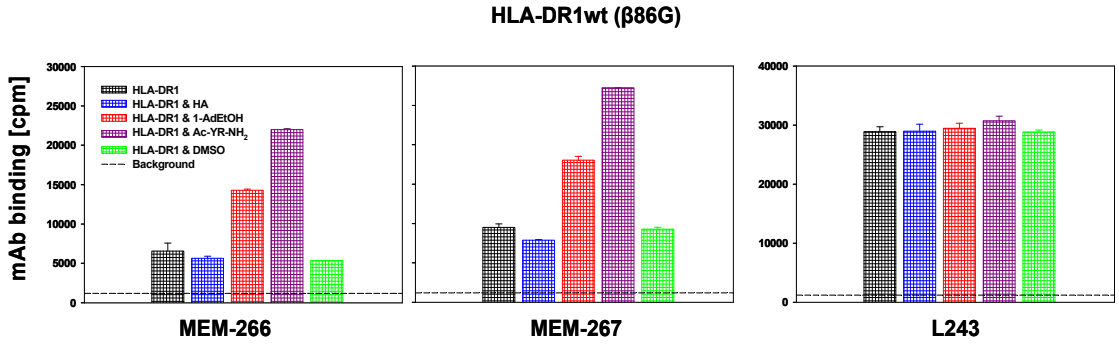

Figure 7: Detection of MLE induced receptive state by MEM-266 antibodies. Similar to the previous experiments soluble recombinant biotin labelled HLA-DR1 molecule were incubated with MLE. Enhanced MEM-266 (figure left), MEM-267 (middle figure) or control L243 (figure right), reactivity was determined. Results are shown in bar chart format as HLA-DR1 (black), HLA-DR1 and HA306-318 peptide (blue), HLA-DR1 and 1-AdEtOH (red), HLA-DR1 and Ac-YR-NH_2 (purple), HLA-DR1 and 1% DMSO control (green), background (dotted line). Values are expressed as counts per minute on Y-axis.

4.2.2.3 Conformational shift can be detected also in defined ligand free HLA-DR1 preparation

To formally confirm that enhanced MEM reactivity is only due to conformational shift in HLA-DR1 and not due to removal of endogenous ligands from HLA-DR molecule, the experiment was repeated with HLA-DR1 produced from *E.coli* expression system. Here HLA-DR1 is refolded after production, therefore the preparation is free from any endogenous ligand. As shown in (Figure 8, left panel) similar result was obtained with *E.coli* produced HLA-DR1 showing enhanced MEM reactivity with MLE compound. Both *E.coli* as well as insect expression system produced HLA-DR1, showed enhancement to MEM-267 antibody reactivity. This effect can also be observed in a dose dependent manner using chemical MLE, 1-AdEtOH and pCP, with more stronger effect observed with 1-AdEtOH, a stronger MLE and less with pCP a weaker MLE (Figure 8, right panel).

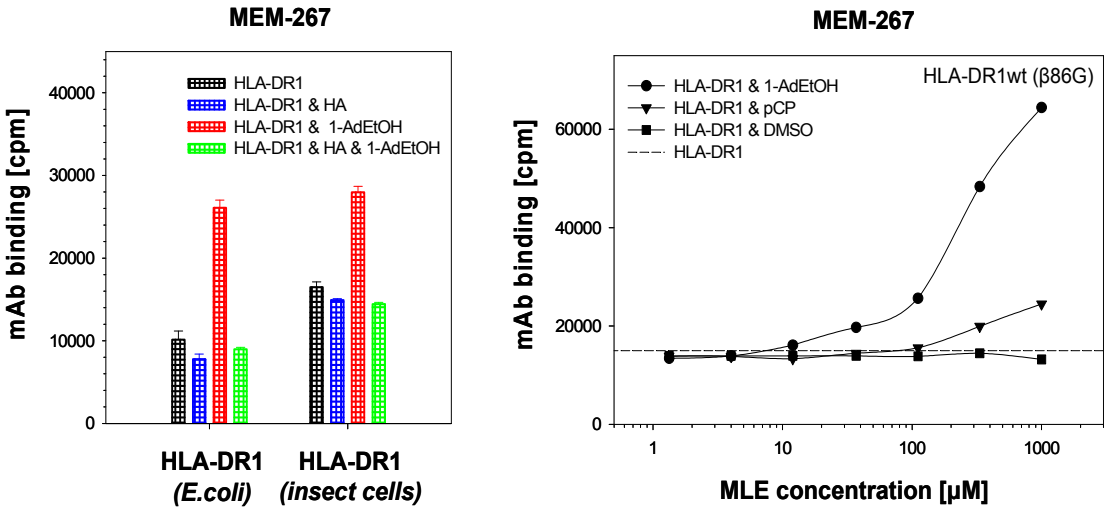

Figure 8: MLE induced enhanced MEM reactivity is only due to conformational change. (Left panel) Experiment was carried out with 150 nM biotin labeled HLA-DR1 produced from *E.coli* expression system (free of endogenous ligand) in comparison to 500nM of insect produced HLA-DR1 (loaded with endogenous ligand from insects), in presence and absence of MLE. The result is summarized in bar chart as HLA-DR1 (black), HLA-DR1 & 50µg/ml HA (blue), HLA-DR1 and 1-AdEtOH (red), HLA-DR1 & 1-AdEtOH & HA (green). Enhanced monoclonal antibody binding was determined by ELISA and values are expressed as counts per minute on Y-axis. (Right panel) MLE induces conformation shift in HLA-DR1 protein in dose dependent manner. Experiment was carried out by incubated 300nM biotin labeled HLA-DR1 protein in presence of titrated amount of chemical MLE 1-AdEtOH (filled circle), pCP (inverted filled triangle) and 1% DMSO control (filled square).

Therefore it was confirmed that enhanced MEM reactivity observed was not due to removal of endogenous ligand but it was only due to conformational change in HLA-DR molecule, which is better recognized by MEM antibodies. As this conformational transition is induction of receptive state due to MLE compounds. Hence these antibodies probe for the *'receptive state'*, rather than only for *'empty'* MHC.

4.2.2.4 MEM antibodies show MLE activity

As the epitope of these antibodies is present in the binding site of the MHC class II molecule and as they are able to detect the receptive state of HLA-DR molecule therefore it was assumed that the antibodies might also stabilize the receptive state. As a result these antibodies might catalyze the antigen loading on MHC class II molecule, behaving as MLE compound. To verify the assumption soluble HLA-DR1 molecule wt (β86G) and mutant HLA-DR molecule with β86G→V, were incubated with ABL 908-922 peptide in presence of titrated amount of MEM 266, 267 antibodies or as a control L243 antibody. While L243 antibody did not shown any enhancement in the antigen loading, MEM antibody catalyzed the antigen loading on HLA-DR1 variant (β86G, β86V). This catalysis observed was found to be dose dependent (Figure 9).

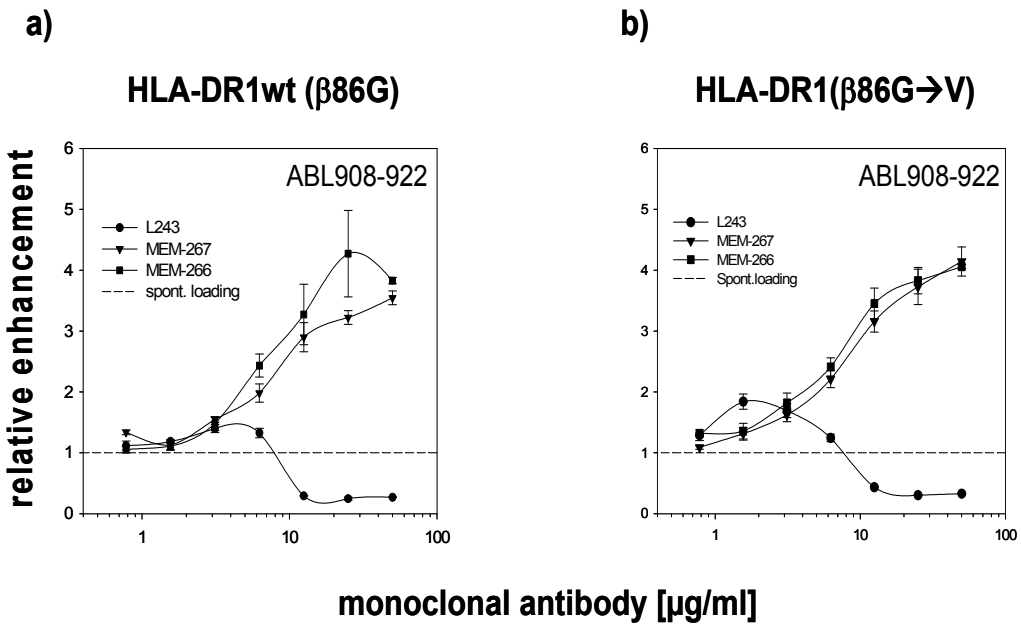

Figure 9: MEM antibodies show MLE effect. To confirm that MEM antibodies might stabilize the receptive state and therefore work as catalyst, HLA-DR1 wt (β86G) (figure left) HLA-DR1 mutant β86G→V (figure right) were incubated with titrated amount of antibodies, MEM-267 (filled inverted triangle), MEM-266 (filled square) or control L243 (filled circle) in presence of 1µg/ml of ABL 908-922 for HLA-DR1 wt (β86G), 5 µg /ml for HLA-DR1 mutant β86G→V. Complex formation is expressed as relative enhancement in reference to the spontaneous complex formation in the absence of any antibody. Spontaneous complex formation without any antibody presence is shown by dashed line.

This section suggests the conformational evidence to support mechanism behind MLE mediated ligand exchange. The spectral experiments could prove the conformational shift occurring in MHC molecule. The enhancement in conformational sensitive antibody reactivity supported the spectral data, and thereby endorse the effect that conformational shift occurring in MHC class II molecule is only due to MLE. The results indicate that these antibodies also detect the '*receptive'* conformation apart from detecting 'empty' MHC. It was interesting to know that the conformational transition induced by MLE are propagated to the far distal end of HLA-DR1 β chain, away from peptide binding site, near to HLA-DM binding residues. Thus hypothesized MLE induced conformational change could be supported experimentally.

4.3 Role of peptide-MLE in celiac disease

4.3.1 Structure activity relationship of dipeptides on HLA-DQ2

In general peptide-MLE could have potential role as a risk factor in inflammatory or auto immune disease. Celiac disease was a suitable model to analyze it. This disease is strictly linked to MHC class II allele, HLA-DQ2 (80-90% patients) and HLA-DQ8 (10-15% patients) (Sollid, 2002). However so far no chemical compounds are known to effect the ligand exchange on HLA-DQ2. Identified HLA-DR1 MLE compounds could not show MLE activity on other MHC class II alleles. However it is universally known that pocket 1 is present in all the MHC class II molecules (Figure 1).

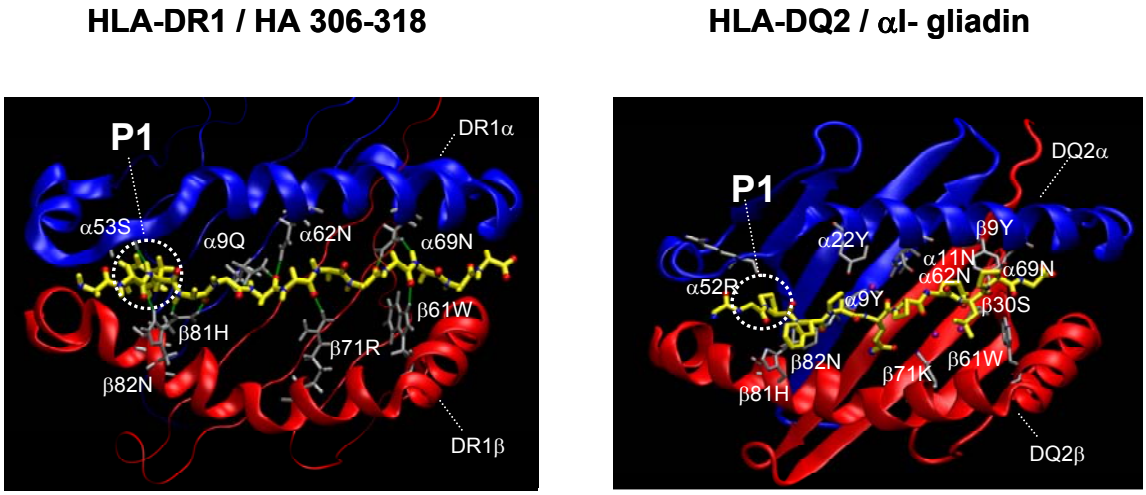

Figure 1: Crystal structure of HLA-DR1 with HA306-318 peptide in comparison to HLA-DQ2 with deamidated α1 gliadin : Top view shows the location of pocket 1, with HA306-318 peptide bound to HLA-DR1 protein (figure left) or gliadin αI peptide bound to HLA-DQ2 protein (figure right). Only the α1 and β1-domain of MHC molecule are depicted. α1 shown by blue ribbon and the β1-domain of the MHC molecule is depicted by red ribbon. Location of P1 pocket is depicted by dotted circle. Peptide backbone of HA306-318 ligand (HLA-DR1) and gliadin αI peptide (HLA-DQ2) is enumerated by yellow colour and MHC residues forming H bond interaction with backbone are labelled with grey. (pdb:1dlh) (pdb: 1s9v) (Gupta, et al., 2008).

Therefore we presumed that due to basic structural similarity between HLA-DR1 and HLA-DQ2, the dipeptides should also show catalytic effect in HLA-DQ2 allele. In order to confirm if the dipeptides can mediate the antigen loading on this MHC class II allele, a set of dipeptides which were previously used for HLA-DR1 were also used for HLA-DQ2. Soluble HLA-DQ2 protein was incubated with a biotinylated MHC Ia 46-60 (high affinity HLA-DQ2 binder indicator peptide) (Vartdal, et al., 1996) in presence and absence of peptide-MLE. In parallel HLA-DR complex formation was carried out with HA306-318. The complex formation was determined by ELISA. Indeed the dipeptides with bulky

aromatic side chain anchors could show better MLE effect. Moreover HLA-DQ2 is known to bind bulkier groups and this is reflected in the loading experiment. Dipeptides with bulkier tryptophan groups showed best enhancement on HLA-DQ2, however Ac-FR-NH_2 was found to be the best MLE on HLA-DR1**.** Order of activity observed was W>Y>F >L for HLA-DQ2 (Figure 2 right panel) while for HLA-DR it was found to be F>W>Y >L (Figure 2 left panel).

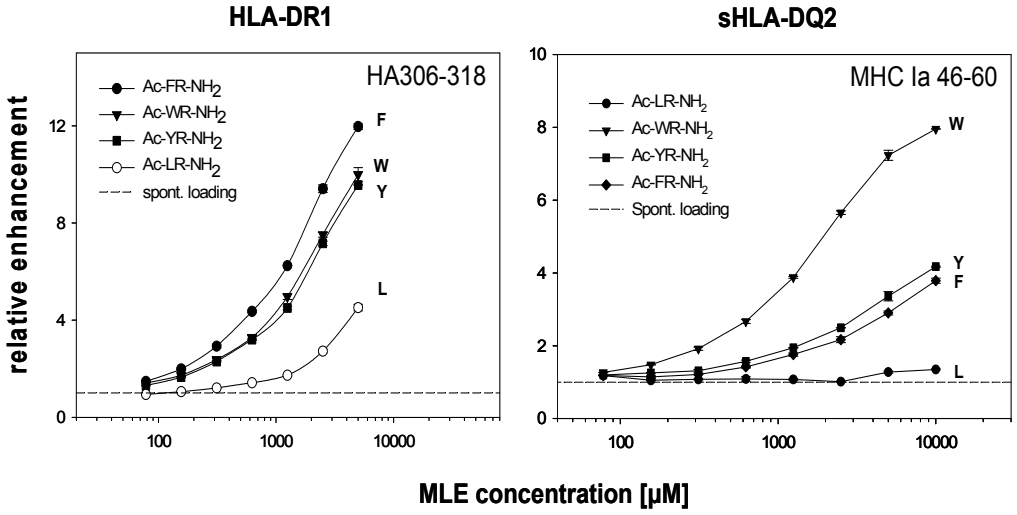

Figure 2: Preference for bulkier groups for HLA-DQ2 in comparison to HLA-DR. Comparison of the catalytic activity of set of aromatic and aliphatic peptide-MLE on HLA-DQ2 in comparison to HLA-DR protein preferences. 400nM of soluble HLA-DQ2 was incubated with 20µg/ml of biotin labelled MHC Ia 46-63 peptide (figure right), or 200nM of HLA-DR1 protein in presence of 50µg/ml of HA306-318 peptide in presence of peptide-MLE (figure left). Enhancement in antigen loading for HLA-DQ2 is shown as Ac-LR-NH_2 (filled circle), Ac-WR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-FR-NH_2 (filled diamond). For HLA-DR Ac-FR-NH_2 (filled circle), Ac-WR-NH_2 (filled triangle down), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (open circle). Peptide/MHC complex formation was determined by ELISA and is expressed as relative enhancement in response to spontaneous complex formation in the absence of catalyst. Spontaneous loading without catalyst is shown by dotted line.

In case of HLA-DR1, N-terminal acetylation and C-terminal amidation enhance the catalytic influence of peptide-MLE. Role of N and C-terminal modifications of dipeptides on HLA-DQ2 was confirmed in an *in vitro* loading assay. Soluble HLA-DQ2 protein was incubated with biotin labelled MHC Ia 46-60 peptide (Vartdal, et al., 1996), in presence of unmodified or N and C-terminal modified dipeptides. In parallel HLA-DR1 soluble protein was also incubated with biotinylated HA306-318 in presence and absence of modified MLE. In contrast to the effect observed with HLA-DR1 where both N-terminal acetylation and C-terminal amidation of YR showed enhancement in MLE effect (Figure 3b). Ac-YR did not show any enhancement in catalytic effect but slight enhancement was observed with C-terminal amidation of YR (YR-NH_2) on HLA-DQ2 (Figure 3a).

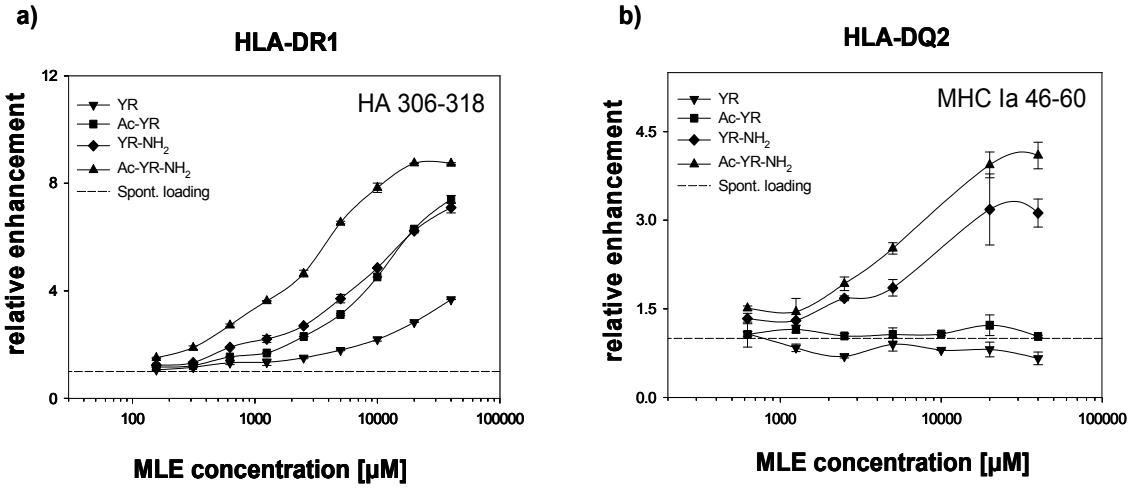

Figure 3: Structure activity relationship of dipeptide affecting celiac disease linked HLA-DQ2 molecule. To compare and validate if dipeptides can also catalyze the peptide loading on HLA-DQ2 and also to validate the impact of N-terminal acetylation and C-terminal amidation on catalytic effect of dipeptides, in comparison to HLA-DR1, *in vitro* peptide loading assay was carried out. 20µg/ml of MHC-Ia 46-60 peptide, was incubated with 400nM of recombinant soluble HLA-DQ2 (figure right) or 200nM of HLA-DR1 protein in presence of 40 µg/ml of HA 306-318 peptide (figure left). The reactions were catalyzed by modified or unmodified dipeptides as shown above. Results are shown as YR (filled inverted triangle), Ac-YR (filled square), YR-NH_2 (filled diamond), Ac-YR-NH_2 (filled triangle up).

Any replacement of the standard L-amino acids by the respective D-enantiomer also abrogated the catalytic activity in case of HLA-DQ2 (data not shown). Moreover any modification of tyrosine (Y) of Ac-YR-NH_2 to β-homotyrosine resulted in the complete loss of activity due to increase in the distance between side chains by an additional CH_2 group present in β-homotyrosine (data not shown). Ac-AR-NH_2 did not show any MLE effect on HLA-DQ2 (data not shown). Stearic requirements H-bond utility and in particular failure of dipeptides lacking the aromatic side chain further support the assumption that the effect might be mediated by pocket 1 in HLA-DQ2.

4.3.2 Dipeptides can catalyze loading of gluten derived antigen

Deamidated 33mer (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF) a α2-gliadin 56-88 peptide generated from digestion of gluten has been previously shown to be a long survivor gluten fragment in the gut. This antigen binds to HLA-DQ2 with very high affinity in celiac disease patients. This is also a potent stimulator of T cell lines obtained from celiac disease patient biopsies. It is recognized much more efficiently by intestinal T-cell lines than shorter peptides covering the DQ2 α-1, α-II, and α-III epitopes (Shan, et al., 2002). HLA-DQ2 protein was incubated with biotinylated deamidated 33mer (33mer.E) in presence of titrated amount of peptide-MLE. Experiments were carried out with HLA-DQ2

protein obtained from different sources either by recombinant method (Figure 4b) (soluble HLA-DQ2) or membrane solubilization of EBV transformed B lymphoblastoid DR3-DQ2 (DQA1*0501/ DAB1*0201) homozygous '9023' VAVY cell line (det. solubilized HLA-DQ2) (Figure 4a).

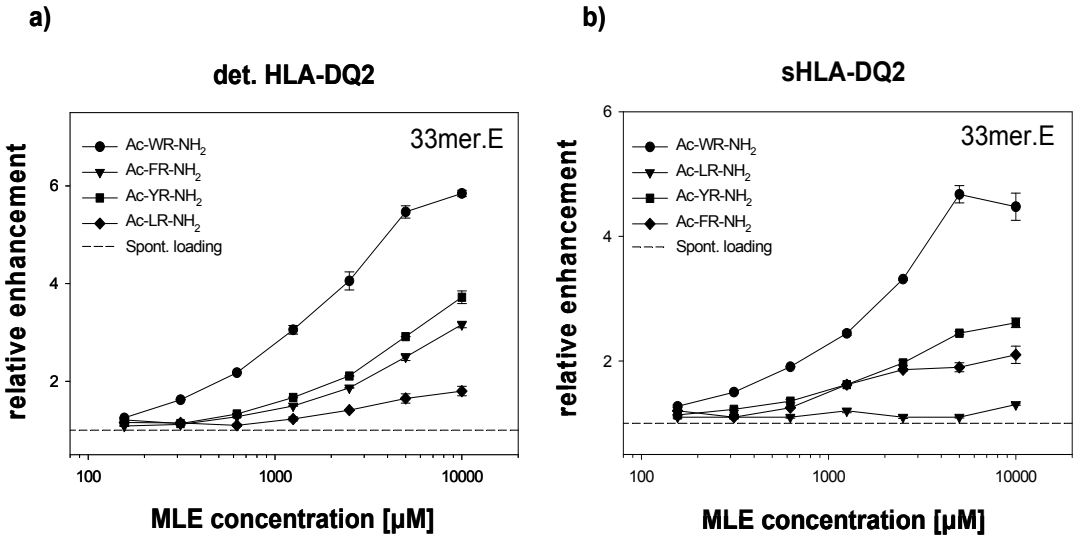

Figure 4: Impact of peptide MLE in enhancing antigen loading on detergent solubilized HLA-DQ2. To verify if dipeptides can enhance the loading of deamidated 33 mer (33mer.E) on detergent soluble DQ2 (figure left), a loading assay was performed in comparison with recombinant sHLA-DQ2 (figure right). Briefly 100nM of det. solubilized HLA-DQ2 or 400nM of sHLA-DQ2 (figure right) were incubated with 200nM of 33mer antigen in presence of titrated amount of catalytic dipeptides. The enhancement in antigen loading for det. solubilized HLA-DQ2 (figure left) is shown as Ac-WR-NH_2 (filled circle), Ac-FR-NH_2 (filled inverted triangle), Ac-YR-NH_2 (filled square), Ac-LR-NH_2 (filled diamond) and for sHLA-DQ2 (figure right) Ac-WR-NH_2 (filled circle), Ac-YR-NH_2 (filled square), Ac-FR-NH_2 (filled diamond), Ac-LR-NH_2 (inverted filled triangle). Complex formation is expressed as relative enhancement in reference to complex formation without any MLE (spontaneous loading). Spontaneous loading is shown by dashed line.

To confirm whether dipeptides could also enhance the loading of other gliadin epitopes γ-II epitope, γ-5 (227-237) with 'E' at 232 (GIIQPEQPAQL) generated from gluten, (Vader, et al., 2003) and recognized by the T cell clones obtained from celiac disease patient (Qiao, et al., 2005).

The experiment was repeated with biotinylated deamidated γ-II epitope (γ-II.E) in presence of peptide-MLE. Dipeptides were able to enhance the loading of γ-II.E epitope (Figure 5a) in a similar manner to deamidated 33mer epitope (Figure 5b). Best catalytic activity was observed with Ac-WR-NH_2 and least activity was shown by Ac-LR-NH_2. Thus not only deamidated 33mer but dipeptides can also enhance the loading of other gluten derived epitope like γ-II.

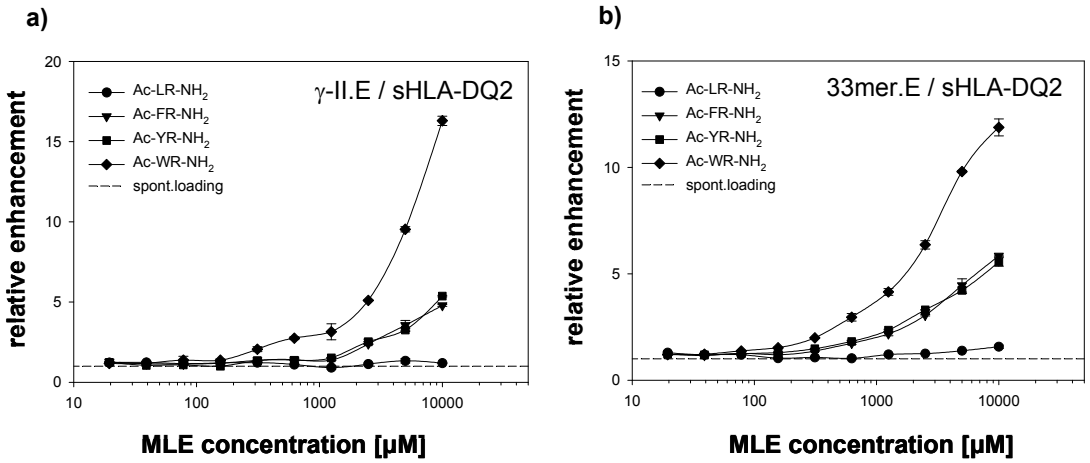

Figure 5: Impact of peptide MLE in enhancement of other gluten derived antigens. To demonstrate that peptide MLE can catalyze the loading of other gluten derived antigens, complex formation was carried out with 300nM of sHLA-DQ2 and 5µg/ml of γ-II.E epitope (figure left) or 200nM of 33mer.E epitope (figure right) in presence of titrated amount of peptide MLE. The result is shown as comparative catalytic effect of peptide MLE in accelerating the antigen loading of both 33mer.E and γ-II.E epitope in same experiment as Ac-LR-NH_2 (filled circle), Ac-FR-NH_2 (filled inverted triangle down), Ac-YR-NH_2 (filled square), Ac-WR-NH_2 (filled diamond). Spontaneous loading without peptide-MLE is shown by dashed line.

4.3.3 Enhanced loading of gluten derived antigen by dipeptides on APC cell surface

The *in vitro* soluble protein experimental data have already shown that peptide-MLE could enhance the gluten antigen loading on soluble HLA-DQ2 protein. To confirm whether this applies to cell surface HLA-DQ2 molecules, APC loading assay was performed. Cell surface antigen loading was analyzed by flow cytometry (FACS). For these experiments EBV cells ('9023' VAVY cells) expressing HLA-DQ2 on the cell surface were used for the experiment. To confirm the binding of the 33 mer epitope on cell surface MHC molecule, '9023' VAVY cells were incubated with titrated amount of biotin labelled 33mer.E peptide (Figure 6a). Subsequently '9023' VAVY cells were incubated with fixed concentration of biotin labelled 33mer.E in presence and absence of titrated amount of peptide-MLE. Quantification of peptide loading by FACS revealed a similar pattern of enhancement as observed before with soluble HLA-DQ2 molecules. Dipeptide can accelerate the deamidated 33mer antigen loading on cell surface expressed HLA-DQ2 molecules (Figure 6b). Maximum enhancement was observed by Ac-WR-NH_2 and minimum was observed with Ac-LR-NH_2 (WR>FR>LR).

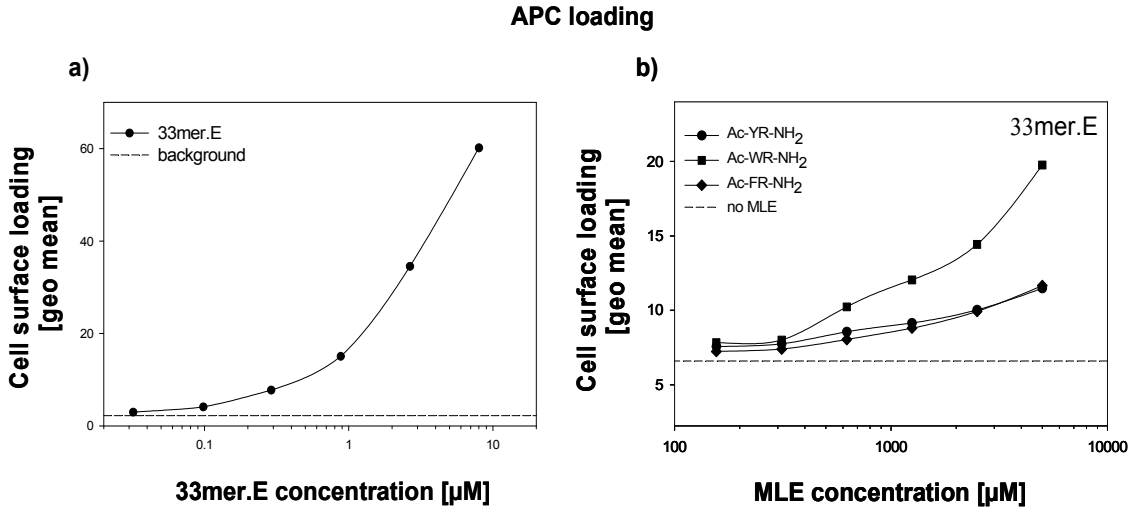

Figure 6: Dipeptides can mediate antigen loading enhancement of deamidated 33mer peptide on cell surface of HLA-DQ2 molecules. '9023'VAVY cells expressing HLA-DQ2 were incubated with titrated amount of biotinylated 33mer.E (filled circle) /20h (figure 5a). In order check the catalytic affect of peptide-MLE, '9023' VAVY cells were incubated with 500nM of biotinylated deamidated 33mer/24h peptide in presence of titrated amount of Ac-YR-NH_2 (filled circle), Ac-WR-NH_2 (filled square), Ac-FR-NH_2 (filled diamond) or in absence of peptide MLE (dotted line). Cell surface loading was analyzed by FACS, and is expressed on Y-axis as geomean.

For confirming similar effect with γ-II.E epitope, binding of epitope in cell surface expressed HLA-DQ2 molecule was checked for either 4h (Figure 7a) or 20h (Figure 7b) and subsequently biotinylated γ-II.E epitope was incubated with VAVY cells in presence of peptide-MLE for either 4h (Figure 7c) or 24h (Figure 7d). Quantification of cell surface antigen loading was done by FACS. With γ-II.E epitope also Ac-WR-NH_2 showed the best effect. The activity observed in the following sequence (WR>YR>FR>LR). Thus even on cell surface MHC molecules, aromatic side chain anchors show maximum effects and aliphatic ones show weaker effects.

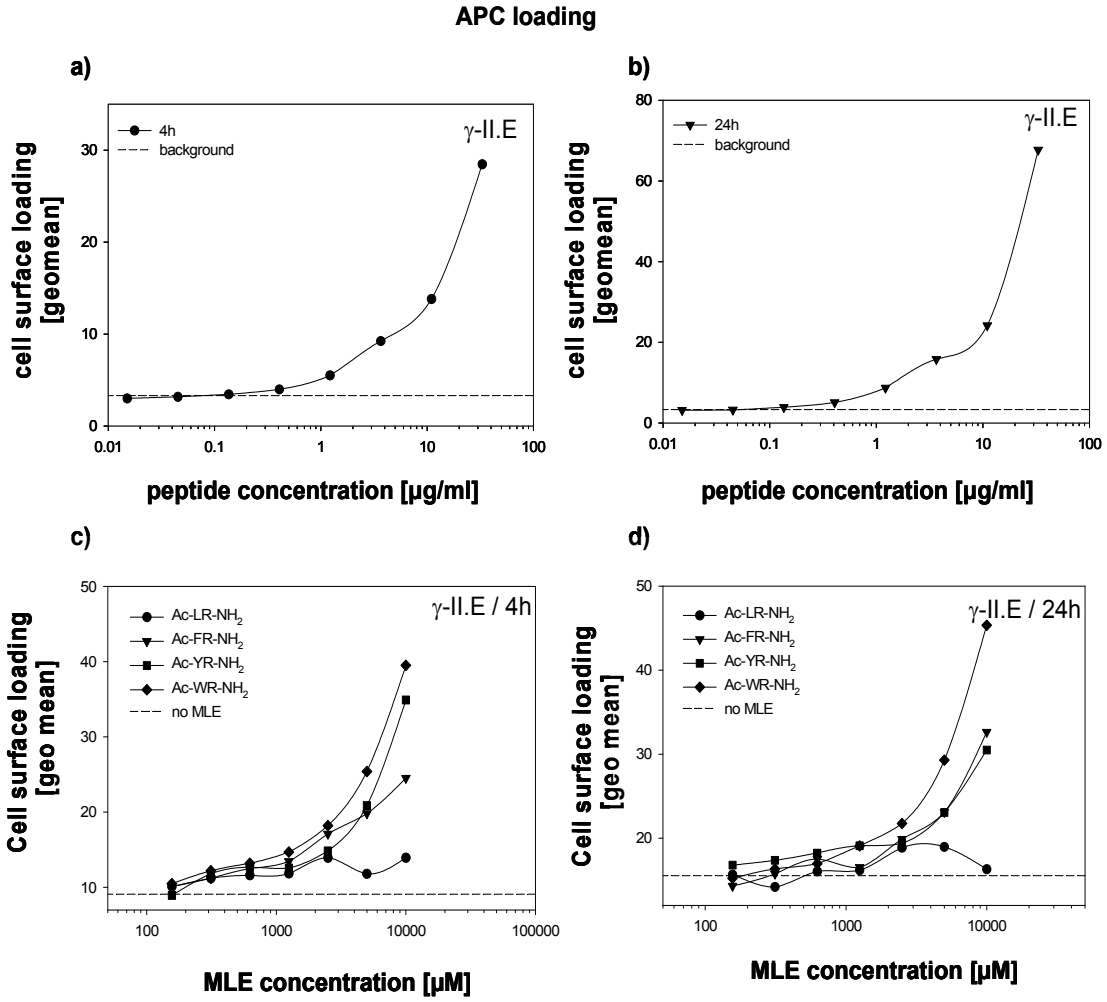

Figure 7: MLE induced enhancement of γ-II.E antigen loading on cell surface of HLA-DQ2 molecules. VAVY cells were incubated with titrated amount of biotinylated γ-II.E antigen for 4h (figure 6a) or 20h (figure 6b), for confirming the binding of epitope on cell surface. In the lower figures, '9023' VAVY cells were incubated with 4µg/ml of biotinylated γ-II.E antigen for 4h (figure 6c) or 24h (figure 6d) in presence and absence of titrated amount of peptide MLE, Ac-LR-NH_2 (filled circle), Ac-FR-NH_2 (filled inverted triangle), Ac-YR-NH_2 (filled square), Ac-WR-NH_2 (filled diamond) or in absence of any peptide-MLE (shown by dashed line). Amount of cell surface antigen loading was determined by FACS, shown as geomean on Y-axis.

4.3.4 Amplification of gliadin specific CD4+ T cell response by peptide-MLE

Uptil now peptide-MLE have been shown in this study to enhance the gliadin antigen loading on soluble HLA-DQ2 as well as cell surface HLA-DQ2 molecules. To confirm that the enhanced antigen loading could be directly translated into amplification of CD4+ T cell response, *in vitro* T cell experiment was performed. The experiments were carried out with

γ-II gliadin specific human T cell clone, Has.1.41 obtained from celiac disease patient intestinal biopsies (Qiao, et al., 2005). EBV transformed B lymphoblastoid cells CD114 (Qiao, et al., 2005) which are homozygous for DR3-DQ2 (DQA1*0501/ DAB1*0201) were used as APC. CD114 cells were incubated with subthreshold concentration of γ-II.E antigen in the presence of titrated amount of peptide-MLE (Figure 8a). Alternatively CD114 cells were incubated with titrated amount of γ-II.E antigen in presence and absence of a fixed concentration of peptide-MLE (Figure 8b). After 24h the APC were presented to HLA-DQ2 restricted and γ-II.E antigen specific T cell clone Has.1.41 (Qiao, et al., 2005). Significant enhancement of T cell proliferation was observed in the presence of peptide-MLE. They could enhance γ-II.E antigen specific response in dose dependent manner (Figure 8a) with maximum proliferation of T cells observed with Ac-WR-NH_2 and minimum with Ac-LR-NH_2. Enhancement in T cell proliferation was observed in following sequence (WR>FR>YR>LR). Presence of Ac-WR-NH_2 could shift the dose response curve to approximately 7 fold as the half maximal response of antigen alone in the absence of catalyst was detected at 60nM, Ac-WR-NH_2 lowered the threshold to 8.5nM. Much weaker effects were observed with Ac-FR-NH_2 and Ac-LR-NH_2 (WR>FR>LR) (Figure 8b).

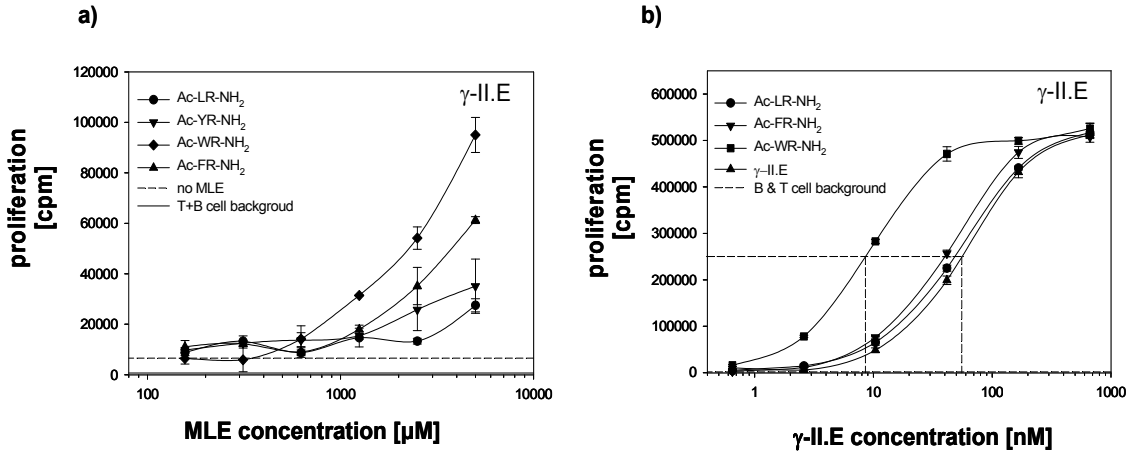

Figure 8: Amplification of γ-II.E gluten antigen specific CD4+ T cell response. The influence of catalytic dipeptides on the antigen specific CD4+ T cell response was tested with T cell clones derived from the intestinal biopsies of HLA-DQ2 positive celiac disease patients. HLA-DQ2 restricted and γ-II.E antigen specific Has.1.41 T cell clone was used for the T cell experiment. CD114 cells were incubated in presence of 12.5nM of γ-II.E antigen with titrated amount of peptide MLE. Ac-LR-NH_2 (filled circle), Ac-YR-NH_2 (filled inverted triangle), Ac-WR-NH_2 (filled diamond), Ac-FR-NH_2 (filled triangle up) or in absence of any peptide-MLE (shown by dashed line), APC T cell background (solid line). In figure right CD114cells were incubated with titrated amount of γ-II.E antigen and fixed 2mM concentration of peptide-MLE. Ac-LR-NH_2 (filled circle), Ac-FR-NH_2 (filled inverted triangle), Ac-WR-NH_2 (filled square), or only γ-II.E (filled triangle up). APC T cell background is shown by dashed line. Dose response curves were generated and T cell proliferative response was determined by addition of thymidine and subsequently reading the counts, data shown as proliferation counts per minute (cpm) Y-axis, concentration of MLE (µM) shown on X-axis (figure 7a) or antigen concentration in nM (figure 7b).

T cell experiments were also carried out with other T cell clones obtained from celiac patient biopsies. T cell clones used for the experiments were TCC 430.1.112 (Arentz-Hansen, et al., 2002) recognizing γ-IV.E γ-5(102 113) 'E' at 106, and 108 (FSQPEQEFPQPQ), a gluten derived antigen (Arentz-Hansen, et al., 2002) or TCC 493.3.4.33 (Arentz-Hansen, et al., 2000) recognizing deamidated 33mer. antigen. CD114 cells were incubated with either titrated amount of γ-IV.E antigen or 33mer.E antigen in presence and absence of fixed peptide-MLE concentration. Respective antigen loaded APC were presented to either TCC 430.1.112 (Figure 9 left) or TCC 493.3.4.33 (Figure 9 right) T cell clones.
Also here enhancement of T cell proliferation was observed in presence of dipeptides. Maximum proliferation of T cells was observed again with Ac-WR-NH_2 and minimum proliferation was observed with Ac-LR-NH_2 (WR>FR>LR). The effect however was substantially weaker as observed with of γ-IV.E antigen.

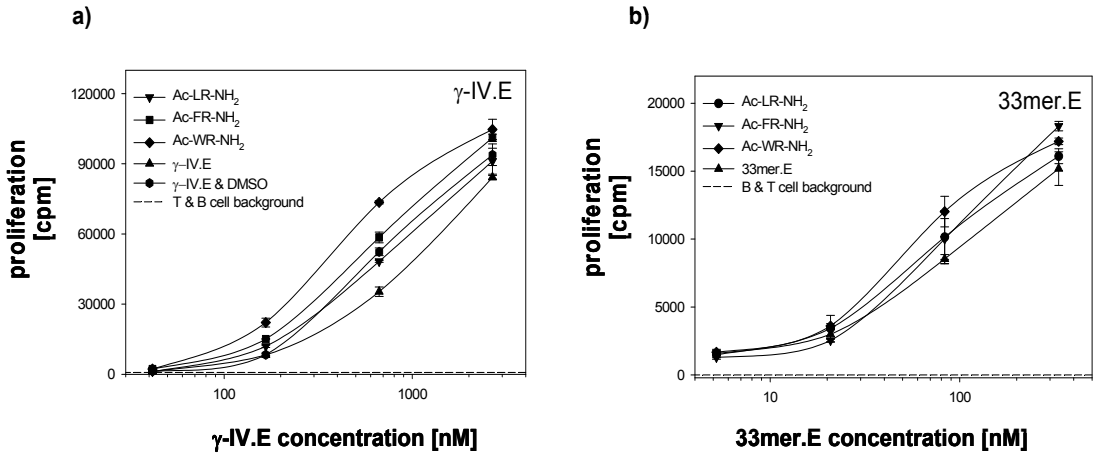

Figure 9: Amplification of 33mer.E or γ-IV.E antigen specific T cell response. Experimental setup was same as mentioned above. Dose response curves were generated by incubating CD114 cells titrated amount of γ-IV.E antigen (figure left) and 33mer.E (figure right) in presence of 2mM peptide-MLE. Subsequently CD114 cells were presented to HLA-DQ2 restricted T cell clones,TCC 430.1.112 specific for γ-IV.E antigen (figure left) or TCC 493.3.4.33 specific for 33mer.E (figure right). MLE responses for γ-IV.E antigen are shown by Ac-LR-NH_2 (filled inverted triangle), Ac-FR-NH_2 (filled square), Ac-WR-NH_2 (filled diamond), γ-IV.E alone (filled triangle up) or γ-IV.E and DMSO control for Ac-WR-NH_2 (filled circle). MLE response for 33mer (figure left) are shown as Ac-LR-NH_2 (filled circle), Ac-FR-NH_2 (filled invertd triangle), Ac-WR-NH_2 (filled diamond), 33mer.E alone (filled triangle up). T and B cell background is shown by dashed line. Dose response curves were generated and T cell proliferative response was determined by addition of thymidine and subsequently reading the counts per minute (cpm), as proliferation.

At least in principle it could be shown here that short peptides can enhance the loading of gluten derived antigen both on soluble as well as cell surface expressed HLA-DQ2 molecules. Ac-WR-NH_2 showed best catalytic effect. The enhanced loading of gluten antigens could be directly translated into amplification of CD4+ T cell responses on T cell clones obtained from celiac disease patients.

5 Outlook

5.1 Therapeutic potential of MLE

The study described in this thesis contributes to the development of *'natural like'* short peptide 'MHC loading enhancers', finding application as vaccine *'adjuvant'* or *'additive'* in various therapeutic settings. *In vitro* it could be shown that antigen specific immune response could be significantly amplified and additionally tumour antigen specific response in *ex vivo* settings could be enhanced. To develop more potent peptide-MLE, optimization of the lead moiety to 'drug like' compound could be done. Studies can be carried out to test the activity of optimized peptide-MLE during *in vivo* conditions and in mouse tumour models. This may eventually lead to phase I study in humans.

5.2 Structural dynamics of MHC molecules

Ultimate goal would be to visually observe hypothetical conformation by spectroscopic approaches, to produce direct structural information. One way would be X-ray crystallization approach, with crystallization in presence of MHC ligands as well as in presence of MLE, which is ongoing in our group. Other approach would be to resolve the structure of HLA-DR by NMR, as being done in our collaborative lab of Dr. Christian Freund. This process is complicated due to large size of the HLA-DR molecule, however distinct spectra for HLA-DR could be obtained.

5.3 MLE as putative environmental risk factor or implication on autoimmune induction

Lastly, short peptide fragments have been shown here to enhance the gluten antigen specific immune response, in relation to HLA-DQ2 linked celiac disease. Peptide-MLE study can also be extended to other autoimmune disorders like Type-I diabetes which is linked to HLA-DQ8 and narcolepsy that is linked to HLA-DQ6. In my preliminary data I could show that the peptide-MLE could enhance the loading of autoantigen GAD peptide, on cell surface HLA-DQ8 molecules (data not shown). On important issue would be to find real potent natural MLE compound. Preliminary data have shown that natural terpenoids could enhance antigen loading on HLA-DR molecules (data not shown). Secondly it would be important to show the effect in animal models, that MLE are able to trigger, 'inflammatory' autoimmune disease. This might provide better information and would fully unravel the effect of MLE to various other inflammatory-'autoimmune disorders' as 'environmental' risk factor.

6 Discussion

This study shows that short peptide fragments can influence the ligand composition of class II MHC molecules in a catalytic way. By placing an amino acid side chain into a defined pocket of the MHC molecule they trigger ligand-exchange and antigen-loading. Mutational analysis indicated already that the occupation of pocket P1 is crucial for the catalytic effect of organic MLE compounds (Hopner, et al., 2006). As demonstrated here for the human HLA-DR1 molecule, a similar role could also be established for the ligand exchange driven by short peptides. Evidence from chemical MLE data suggested, that mere filling of P1 might stabilize the 'receptive' conformation. Therefore dipeptides were rationally designed, specifically to target this pocket.

Pocket 1 is supposed to be key structural element of MHC class II molecule and is present in all MHC class II molecules. While the location of the pocket is conserved, it contains polymorphic residues that determine allele-specific preferences for anchor residues (Stern, et al., 1994). Since the same structural requirements also dictate the interaction with 'catalytic' side chains of short peptides, they exhibit their effect in an allele-selective way. The experimental data showed clear correlation between the catalytic activity of MLE to the known anchor preference for HLA-DR1 molecule. Site directed mutagenesis confirmed that the dipeptide target P1 pocket.

Studies showed already that the substitution of residue β86G by tyrosine resulted in a 'filled' P1 pocket and produced an MHC molecule with elevated receptiveness (Chou and Sadegh-Nasseri, 2000). P1 is located proximal to the binding site of natural catalyst HLA-DM and binding studies suggested that HLA-DM interacts specifically with the flexible empty hydrophobic P1-pocket (Chou and Sadegh-Nasseri, 2000). While the active conversion of a non-receptive molecule by HLA-DM has recently been questioned (Grotenbreg, et al., 2007), it is undisputed that the chaperone interacts with a region proximal to P1 to stabilize the peptide receptive conformation (Denzin, et al., 1996; Doebele, et al., 2000; Kropshofer, et al., 1997; Narayan, et al., 2007).

While inside the cell the occupational state of P1 seems to control the interaction with key-components of the processing pathway, on the cell surface it may regulate the transition into the 'non-receptive' conformation. Here, it functions as trigger for a safeguard mechanism that closes the binding site as soon as the ligand is lost. In this study it is shown that small peptide fragments can by-pass this mechanism in a catalytic way. Particularly striking is the effect on the ligand exchange. Peptide-MLE were able to increase not only the loading of empty HLA-DR molecules but also the dissociation of HLA-DR molecules preloaded with lower affinity ligands. The activity of these peptide-MLE were found to be highly stereospecific with only natural L-aa conformer showing activity and D-aa conformers were inactive. Moreover any increase in the backbone length by introducing additional carbon atom on the MLE leads to the disruption of the catalytic effect. This unique specificity of our designed dipeptides show drug like nature and also point towards optimum utilization of H-bond network around P1 pocket.

Peptide-MLE were infact specially designed to utilize natural H-bond network of the MHC to place the pharmacophore into the right spot P1. Additional modifications in the dipeptides to utilize the natural Hydrogen (H-) bond network in the form of acetylation and amidation did therefore enhance the MLE activity on HLA-DR1. While on HLA-DR1 both

N-terminal acetylation and C-terminal amidation did increase the activity. The experiments involving HLA-DQ2 did not show any enhancement in MLE activity with N-terminal acetylation. This however is in line to expectation that the H- bond donor at α-53 is missing in HLA-DQ2 (Kim, et al., 2004). The result confirms the importance of H- bonds, and also suggest that in near future some peptide hybrids could be made to develop a better MLE compound. Peptide backbone being a natural carrier, and modifications can be offered on the side chain, to design a more potent MLE.

Similarly to HLA-DM our dipeptides were unable to enhance antigen loading on mutant HLA-DR molecule, with P1 pocket blocked by bulky tyrosine residue (β86Y). While pCP a small molecule specific for HLA-DR, having activity independent of dimorphic residue β86 could also enhance the loading on this mutant HLA-DR molecule. This suggests that apart from P1, other sites could also be targeted for MLE effect. 'Ii chain-key' peptides (Chou, et al., 2008; Chou and Sadegh-Nasseri, 2000; Humphrey, et al., 1996) have already been shown to enhance antigen loading by targeting allosteric site located near the N-terminal site of MHC molecule. On experimental verification no co-operative effect of these 'Ii chain-key' peptides were seen in presence of our dipeptides. Moreover activity of modified 'Ii-key' peptides revealed similar preference for the catalytic side chain as required for P1 anchor residue (data not shown). This might also question the existence of specific 'allosteric site' for Ii-key peptides which most likely also target same P1 pocket.

From the previous model it was presumed that Pocket 1, opening is stabilised by MLE compounds (Hopner, et al., 2006). This is further supported by a new computational model based on an unbiased calculation. Here it was observed that the transition to the non-receptive state is directly correlated with structural changes inside pocket P1 (Gupta, et al., 2008). The direct correlation of the catalytic activity with the structural requirements of P1 introduced by this study provides additional support to the hypothesis that the stabilization of P1 prevents the transition into the 'non-receptive state'. The molecular dynamic (MD) simulation confirmed that the pocket P1 is indeed quickly lost when the peptide ligand is stripped off from the MHC molecule (Figure 1). Moreover calculations based on the coordinates of the crystal structure of the HLA-DR1/HA306-318 complex revealed that the most significant transitions were detected near the P1 pocket. While these shifts resulted in a narrowing of the two α-helices by more than 7A° (Figure 1A), they also led to a complete loss of the P1 pocket. In less than 15 ns the P1 cavity was filled with side chains or removed by distortions (Figure1 C,D). Notably, this collapse was prevented when prior to the MD simulation the Ac-FR-NH_2 was docked into the P1 pocket (Figure 1B).

Based on this model even the partial occupation of the binding site by a very short peptide is sufficient to stabilize the receptive state as long as it positions an anchor side chain inside the P1-pocket. P1 therefore seems to function as a sensor for the peptide load where occupation leads to a stabilization of the 'open' conformation required to accommodate the peptide ligand. Studies by other groups have already shown that the loading status of P1 plays a crucial role as indicator in the intracellular antigen-processing pathway. The interaction with HLA-DM seems to depend on the loading state (Chou and Sadegh-Nasseri, 2000). Its catalytic activity was reported to be mediated by β81 His, a conserved residue on top of P1 pocket (Narayan, et al., 2007).

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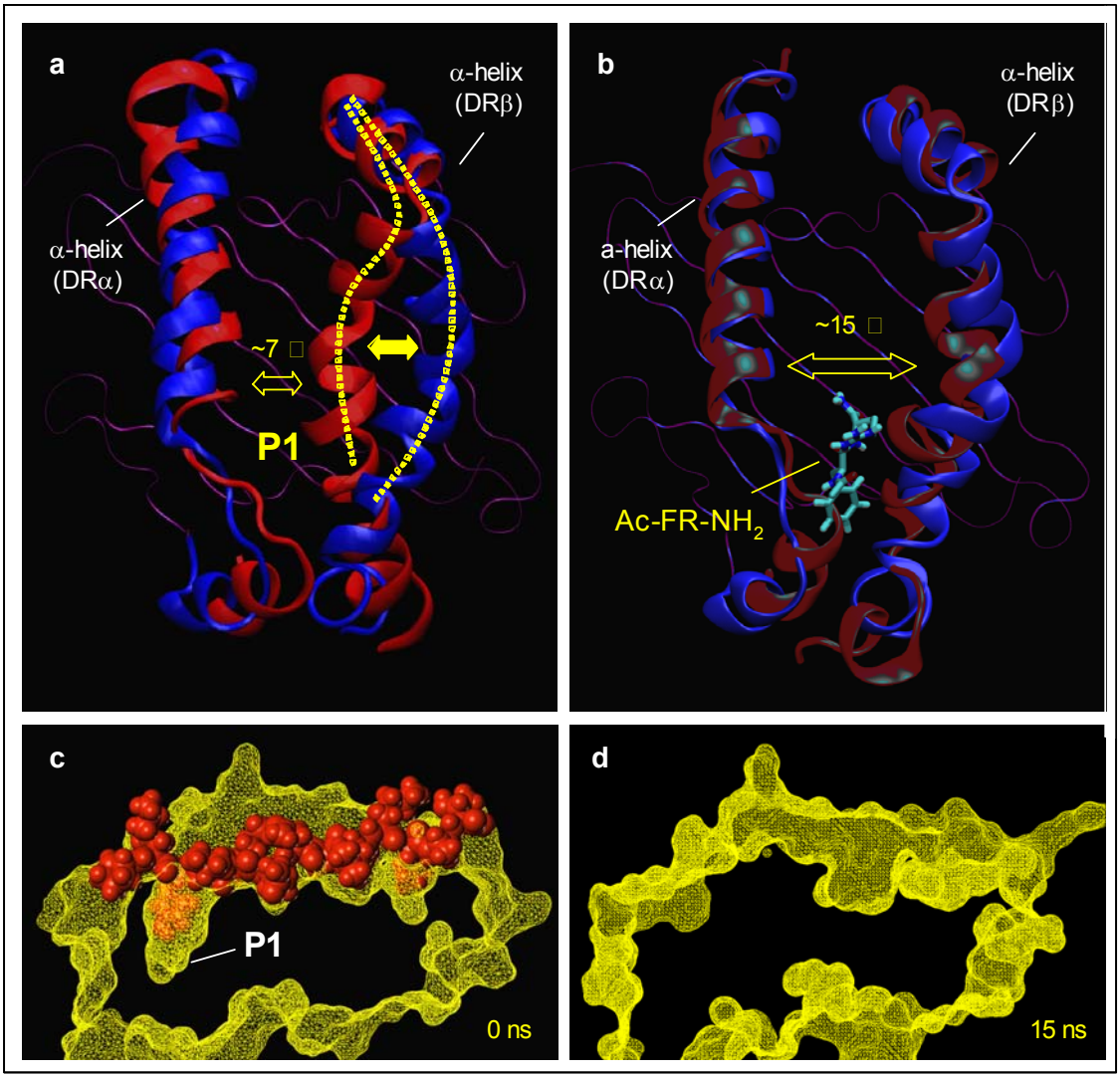

Figure 1. Molecular dynamic (MD) calculation of 'empty' and peptide-MLE stabilized HLA-DR1. The coordinates of the MHC component of the crystallized HLA-DR1/HA306-318 complex (pdb:1dlh) were used to carry out a 15 ns MD calculation with an 'empty' MHC molecule. a) Dynamic of the empty MHC molecule. The floor composed of the β-plated sheats is depicted in magenta, the α-helices of the starting structure are shown in red, α-helices of the structure obtained after 15 ns are shown in blue. The approximate position of P1 is indicated. While the dynamic was carried out with all extracellular domains, only the binding site is shown (α1-, β1-domain). b) Dynamic of the peptide-MLE stabilized MHC molecule. The same MD calculation was carried out as in Fig.1a except that coordinates of an HLA-DR1 molecule were used, in which prior to the MD calculation the peptide-MLE Ac-FR-NH_2 was docked into the P1 pocket. c) P1 pocket in the peptide loaded MHC complex. The image shows a cross-section of the HLA-DR1/HA306-318 complex. The surface of the MHC molecule is shown in yellow, the peptide ligand in red; position of the P1 pocket is indicated. d) Loss of P1 in the empty MHC molecule. The same cross-section shown in Fig. 1c for the peptide-loaded MHC is shown here for the empty molecule obtained after 15 ns of MD calculation. In this structure the P1 pocket can no longer be located. (Gupta, et al., 2008)

To consolidate the computational model, MLE mediated structural transitions were studied in detail using spectroscopic techniques. Data obtained from the ANS binding measurements indicated the stabilization of the 'receptive conformation' which is either quickly lost or remains undetected. Conformational shift were only observed with the MLE susceptible HLA-DR allele. Similar to this the relative shift in the distance of tryptophan residues was only evident when susceptible HLA-DR allele was used. Due to inherent auto-fluorescence of peptide-MLE the spectral studies were restricted to the chemical-MLE compound 1-Adamantane ethanol. The trend of data obtained from ANS as well as intrinsic tryptophan measurement indicated the stabilization of conformation that is receptive to antigen loading, which is the first physical evidence for the conformational transition.

In continuation the structural transitions were also detected during the binding of conformation sensitive antibodies. Binding of the conformation sensitive antibodies to HLA-DR were increased during the presence of either chemical-MLE or peptide-MLE. The result indicated that the antibodies not only detect 'empty MHC molecules' but rather bind specifically to the 'receptive conformation'. This was in contrast to the previous published results where the binding was shown to be reduced in the presence of Ac-YR-NH_2 (Carven, et al., 2004), which might be due to the fact that the previous study was not carried out in serum free medium. Most possible explanation for reduction in the antibody binding, might be due to catalysis of free endogenous ligands present in serum, by the dipeptide that resulted into reduced antibody binding.

The epitope of conformational sensitive antibody MEM-267 (Carven, et al., 2004) and KL-295, KL-304 (Sato, et al., 2000) are near to the peptide binding site in the helical part of the HLA-DR β chain. The experimental data could be correlated with the computational (Bernd Rupp unpublished results). Angle distribution of the helical region forming the epitope were calculated using molecular dynamic (MD) calculation. For the analysis leucine 53 (L53), aspartate 57 (D57), serine 63 (S63), leucine 67 (L67) residues located in the HLA-DR1β chain were used, as these residues are reported to be recognized by the conformation specific antibodies (Carven, et al., 2004). Due to spacing of three to four residues between the above mentioned amino acids, they form the characteristic helical repeat. However in the HLA-DR1 peptide complexes, these residues are not adjacent due to kinks formed in the helical part of the β chain. This non-adjacent distribution of the key epitope residues in the HLA-DR1/HA 306-318 structure suggests that conformational shift is required for the antibody binding to empty or peptide loaded HLA-DR1. With the Cα co-ordinates of these key MEM residues two axis were constructed (Figure 2b). These angle between these axes were measured (angle θ), and marked with the cyan arrow (Figure 2a). Angle distribution observed with receptive HLA-DR1 (β86G→Y) molecule was same as the observed with MLE stabilized HLA-DR1 molecule with angle distribution around 32°. Peptide free HLA-DR1 alone showed a splitted distribution with a maximum at 32° and a second maximum at 49°. Angle distribution of 38° was observed for HLA-DR1 and HA306-318 complex. The results confirm our experimental observation that MEM antibodies detect the 'receptive conformation' of HLA-DR1 molecules.

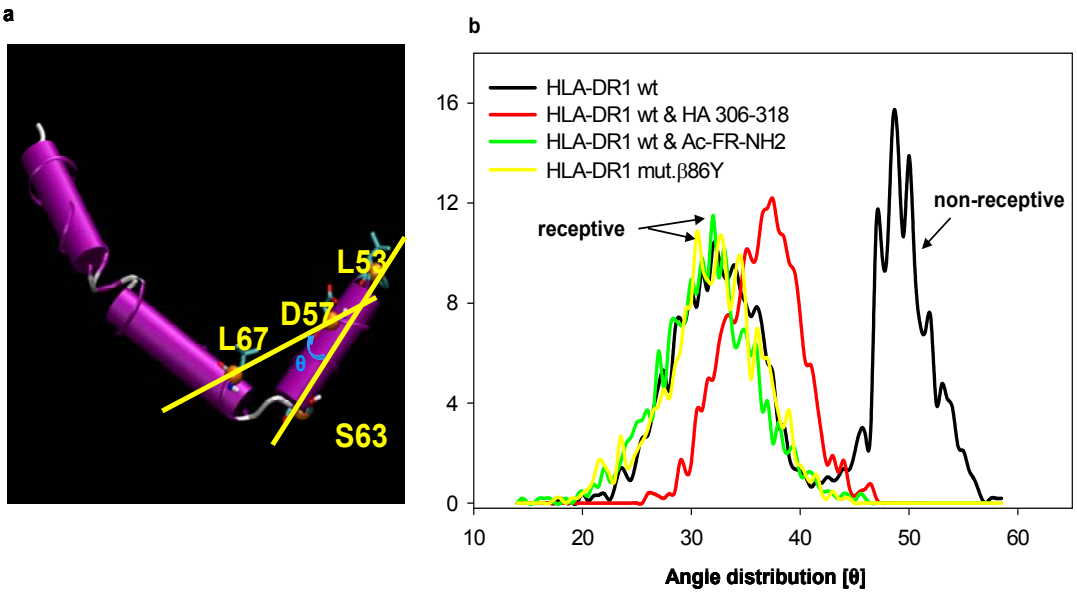

Figure 2 : Molecular dynamic (MD) calculation for measuring angle distribution of MEM epitopes. a) HLA-DR1β chain (purple cylinders) containing MEM epitopes leucine 53 (L53), aspartate 57 (D57), serine 63 (S63), leucine 67 (L67) residues are shown in yellow color. The axes construction by these residues is also shown in yellow color. The angle formed between these axes is shown by cyan color. b) measurement of the angle distribution. Molecular dynamic calculations were carried out for HLA-DR1 wt (β86G) alone, HLA-DR1 wt and HA306-318, HLA-DR1 wt and Ac-FR-NH_2 and HLA-DR1 mutant (β86G→Y). The calculations were performed for 15ns for all except for HLA-DR1 wt (β86G) alone, for which the calculations were performed for 30ns because of slower equilibrium. The results obtained after MD simulation are plotted in the form of curve and are shown as HLA-DR1 wt (β86G) alone in black curve, HLA-DR1 wt and HA306-318 in red curve, HLA-DR1 wt and Ac-FR-NH_2 in green curve and HLA-DR1 mutant (β86G→Y) in yellow curve (Rupp, unpublished)

The MEM data might suggest that that movement might be solely due to sole MLE interaction with antibody or could be only because of conformational rearrangement in MHC molecule, in presence of MLE. It was interesting to observe that the conformational shifts nicely propogated to the C-terminal end of HLA-DR β-chain, away from peptide binding site, as probed by MEM-266 antibodies. Residues near to this region are known to interact with HLA-DM (Doebele, et al., 2000). The results indicate that MLE mediated conformational changes in HLA-DR molecules propagate from the antigen binding site to the distal end of HLA-DR β-chain. Therefore it is equally possible that the inter protein interaction in the particular area could be transmitted to the binding groove. This might in turn regulate the antigen loading and release.

Receptive conformation of MHC molecule has always been a debatable topic as it has only been characterized by kinetic studies. Lack of crystal structure of 'receptive' MHC-II molecule restricts our knowledge regarding the structural information. Moreover we have also identified certain key residues in MHC class II molecules that form a lock, turning HLA-DR, non-receptive to peptide loading (Rupp, unpublished). Recently model of

peptide free HLA-DR1 conformation (Painter, et al., 2008) and HLA-DR3 conformation has been reported (Yaneva, et al., 2009).

The dipeptides in principle are *'natural like'* moeties, which might play important role in under normal or pathological conditions. As shown in this study the antigen presenting cells expressing HLA-DR were susceptible to MLE induced antigen loading. This applies also for dendritic cells. Peptide-MLE were able to enhance the antigen loading on bone marrow derived dendritic cells (BM-DC) obtained from HLA-DR1 transgenic mice.

The enhanced antigen loading on APC was directly translated into amplification of CD4+ T cell responses. The presence of Ac-FR-NH_2 shifted the antigen dose response to nearly 50 fold. Peptide-MLE were able to amplify HA306-318, a CD4+ T cell influenza epitope and also ABL908-922 a newly defined tumor associated antigen obtained from ABL kinase, specific immune response (Gupta, et al., 2008). *Ex vivo* response for NY-ESO-1 89-101, a CD4+ T cell epitope derived from the NY-ESO-1 protein associated with various solid tumors were enhanced during challenge of HLA-DR1 transgenic mice lymph nodes, with Ac-FR-NH_2. The enhancement was detected by robust T cell hybridomas, human T cell lines as well as primary T cells obtained from primed mice expressing human MHC class II. Additionally, 1-Adamantane ethanol, a chemical MLE could enhance antigen specific immune response *in vivo*, in HLA-DR1 transgenic mouse model (Hopner, unpublished).
Peptides generated in endosomes, natural protein fragments present in blood or lymph may also act like peptide-MLE. One abundant source of peptides is human gut. For celiac disease antigen priming takes place in the gut, as a result of direct contact of APC to antigen and peptides. Therefore the study was extended to celiac disease (CD) model. CD is strongly linked to HLA-DQ2 (80%-90%) allele, and is caused due to intolerance to food gluten. By rational design dipeptide, was identified, which could enhance the antigen loading of celiac disease linked-HLA-DQ2. For deep pocket 1 of HLA-DQ2, tryptophan was identified as better anchor. In contrast to HLA-DR loading Ac-WR-NH_2 showed maximum catalytic effect on HLA-DQ2.

Gluten derived epitopes 33 mer (a potent stimulator of T cell lines derived from celiac patients) and γ-II gliadin, loading was significantly enhanced on cell surface HLA-DQ2 molecules. The set of peptide-MLE were also able to enhance the proliferation of gluten epitope specific, CD4+ T cell responses, of clones obtained from celiac disease patient biopsies. Maximum enhancement was observed with γ-II gliadin antigen.

Degradation of proteins results into production of di- and tri-peptides, which are further degraded to single amino acids by peptidases. Our experimental data suggest that various derivatised di-peptides and tri-peptides (data not shown) are able to influence the cell surface ligand repertoire of HLA-DQ2. Moreover these short peptides are able to lower the threshold of antigen recognition or enhance the severity of T cell proliferation. It known that intestinal dendritic cells can penetrate gut epithelia and expose their dendrites inside the lumen of the gut to extremely high polypeptide concentrations originating from diet or commensal debris (Rescigno, et al., 2001). Though currently we do not have any experimental proof but it might be possible that the loading of gluten antigen might be catalyzed by these short peptides, or naturally generated peptide derivatives which might enhance the immune response to gluten derived antigens.

In principle the gluten specific T cell responses could be enhanced by the dipeptides, the concentration required were relatively high. It is known that in human intestine there are 10^{14} commensals comprising more than 400 species. They live on the food that we ingest. (Ogra, 1999). In fact, we allow these bacteria to live and use their fermentation products as nutrients, as stimulators of intestinal absorption and as protective agents against pathogens and cancer (Guarner and Malagelada, 2003; Hooper and Gordon, 2001; Ogra, 1999). Similarly other bio-transformed products, peptide derivatives, produced by the commensals in the gut, or natural products may be identified that might directly modulate the antigen loading on these disease linked HLA-DQ2 allele. By screening of herbal compound library natural terpenoids have already been identified that can catalyze the antigen loading on HLA-DR1 molecule. Enhanced antigen loading translated into amplification of antigen specific CD4+ T cell response during *in vitro* settings (data not shown). Also here absolute chemical compounds might be identified, that might optimally fill pocket 1 of HLA-DQ2. This study atleast suggests that these 'natural like' short peptide derivatives may work as putative risk factors in disease precipitation. Peptide-MLE are able to enhance pathologic reaction involved in autoimmune or inflammatory disease.

While the importance of CD4+ T cells for productive tumour immune responses has just begun to be fully discovered (Corthay, et al., 2005; Wieder, et al., 2008) their role in the induction of autoimmune responses has long been acknowledged (Jones, et al., 2006; Singh, et al., 2008). It is evident for instance in the strong genetic link to class II MHC molecules and in the fact that experimental autoimmune diseases can often be induced by the adoptive transfer of auto-aggressive CD4+ T cells. In this respect 'accidental' loading of these cells with self-antigens by peptide-MLE may therefore trigger unwanted auto-aggressive responses. *In vitro* we have shown already that the presence of simple organic MLE compounds can enhance encephalitogenic T cell responses (Hopner, et al., 2006; Marin-Esteban, et al., 2004). Also *in vivo* severity of the disease has been shown to increase in EAE (experimental autoimmune encephalomyelitis) mouse model, (a model for human multiple sclerosis) in presence of 1-AdEtOH (Dickhaut, unpublished). The same may also apply for celiac disease, where the principle effect could be shown in this study. Capture of soluble antigens by immature DC from lymph or blood, on the other hand, also seems to be an important mechanism for tolerance induction (Hochweller, et al., 2006) and direct cell surface loading has been discussed as an alternative processing pathway of immature dendritic cells (Santambrogio, et al., 1999; Santambrogio, et al., 1999). Natural protein fragments present in blood or lymph acting as peptide-MLE may therefore participate in this process by mediating the direct transfer of antigens onto cell surface MHC molecules. To fully investigate the function of peptide-MLE, studies might be carried out in *in vivo* models to determine the natural role of MLE molecules in immune modulation.
As molecular tool MLE compounds may therefore find applications in experimental and therapeutic settings in which improved antigen loading is desired. A particular suitable field may be peptide-based tumour immune interventions, where the exposure of antigen to a hostile proteolytic environment is extended by the limited access to 'receptive' MHC molecules on the surface of professional APC. In near future MLE might be used as drug like vaccine '*adjuvant*' or '*additive*' in various therapeutic settings. The role of peptide-MLE however is still open and future studies might help in studying MLE function in detail.

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Appendix

Abbreviations

abs: antibodies
1-AdEtOH: 1-adamantane ethanol
Ahx: Aminohexanoic acid
ANS: 8-anilino napthalene sulphonic acid
APC: Antigen presenting cell
Biot: Biotin labeled
β2m: β2microglobulin
BM-DC: Bone marrow derived dendritic cells
CBD: chronic beryllium disease
CD: Celiac disease
CLIP: Class II invariant chain peptide
CTLA4: Cytotoxic T lymphocyte antigen-4
DC: Dendritic cell
DMSO: Dimethyl sulfoxide
EAE: Experimental autoimmune encephalomyelitis
EBV: Epstein Barr Virus
ELISA: Enzyme-linked immunosorbent assay
ELISPOT: Enzyme-linked immunosorbent spot assay
ER: Endoplasmic reticulum
ERAP: Endoplasmic associated amino peptidases
FACS: Fluorescence activated cell-sorting
FITC: Fluorscein isothiocyanate
GFP: Green fluorescent protein
GM-CSF: Granulocyte macrophage colony stimulating factor
HA: Haemaglutinin antigen
His: Histidine
HLA: Human leucocyte antigen
ICAM: Intracellular adhesion molecule
IFN-γ: Interferon-γ
Ii: Invariant chain
IL-2: Interleukin-2
LFA: Lymphocyte function associated antigen
MD: Molecular dynamics
MDC: Max Delbrueck Centre
MHC: Major histocompatibility complex
MIIC: MHC class II compartments
MLE: MHC loading enhancer
mut: Mutant
NK cells: Natural killer cells
P1: Pocket 1
PBS: Phosphate buffer saline
pCP: Parachlorophenol
PDI: Protein disulfide isomerase
PE: Phycoerythrin

SA: Streptavidin
SLE: Systemic lupus erythematosus
TAP: Transporter associated with antigen processing
TCC: T cell clone
TCR: T cell antigen receptor
TG2: Transglutaminase 2
Th1: T helper1
Th2: T helper 2
Tregs: Regulatory T cells
tTG: Tissue transglutaminase
wt: Wildtype
33mer E: Deamidated 33 mer α2 gliadin peptide

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List of publications

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Hopner, S., K. Dickhaut, M. Hofstaetter, H. Kraemer, D. Ruckerl, J.A. Soederhall, S. Gupta, V.M. Esteban, H. Kuhne, C. Freund, G. Jung, K. Falk, and O. Rotzschke. Small organic compounds enhance antigen loading of class II major histocompatibility complex proteins by targeting the polymorphic P1 pocket. *J Biol Chem. 2006 Dec 15; 281 (50): 38535-42.*

Chopra, P., S. Gupta, S.G. Dastidar, and A. Ray. Development of cell death- based method for the selectivity screening of caspase-1 inhibitors. *(manuscript submitted)*

Gupta, S., B. Rupp, S. Gunther, A Shlundt, R. Kuhne, C Freund, K.H. Wiesmuller, G. Jung, K. Falk, and O. Rotzschke. Detection of pocket 1 mediated conformational transitions of HLA-DR. *(manuscript in preparation)*

Gupta, S., U Juse, S.W. Qiao, B. Rupp, L.M. Sollid, R. Kuhne, K. Falk, and O. Rotzschke. Short peptides as ligand-exchange catalyst for celiac disease related gluten peptides. *(manuscript in preparation)*

Rupp, B., S. Gunther, T. Makhmoor, S. Gupta, A. Shlundt, K. Dickhaut, K.H. Wiesmuller, G. Jung, C Freund, K. Falk, O. Rotzschke, and R. Kuhne. A conserved locking mechanism controlling the peptide receptiveness of HLA-DR molecules. *(manuscript in preparation)*

Chopra. P., O. Kulkarni, S. Gupta, M. Bajpai, M. Banerjee, V. Kanoje, T. Chaira, R. Shirumalla, A.K. Verma, V.P. Palle, V.K. Ramanathan, G. Sharma, and A. Ray. Pharmacological profile of AW-814141, a potent orally active inhibitor of p38 MAP kinase. *(manuscript in preparation)*

Oral presentations

Impact of small molecules on antigen presentation by celiac disease associated HLA-DQ2, presented at Leiden University Medical Centre (LUMC) Leiden, Netherlands in *November 2005.*
Catalytic effect of peptide-MHC Loading Enhancer, on HLA-DQ2 presented at EMC Microcollections GmBH Tübingen, Germany in *October 2006*.
Catalytic effects of MLE on celiac disease linked HLA-DQ2 molecule presented at Institute Curie, Paris France in *October 2006.*
Small molecule as ligand Exchange catalyst on Celiac Disease linked HLA-DQ2 presented at Leiden University Medical Centre (LUMC) Leiden, Netherlands in *March 2007.*
Catalytic effect of peptide-MHC loading enhancers on HLA-DR presented at Forschung Institute for Molecular Pharmacology (FMP) in *November 2007*
Anchor side chains of short peptide fragments trigger ligand exchange of class II MHC molecules presented at Institute of Immunology, Rikshospitalet, Oslo in *March 2008.*

Poster

Gupta S., S. Hoepner, K. Dickhaut, B. Rupp, R. Kuhne, C. Freund, G. Jung, K. Falk, and O. Rotzschke. Small molecular MHC-loading enhancers (MLE) amplify immune response by targeting pocket 1 of class II MHC proteins. at 94th Annual Meeting of American Association of Immunologists at Miami, Florida USA, May 18-22, 2007.

Place: Berlin Shashank Gupta

Date: 15 January 2009

Eidesstattliche Erkläerung

Hiermit erkläre ich, die vorliegene Arbeit selbstaendig angefertig zu haben. Ich habe keine unerlaubten sowie unerwaehnten Hilfen benutzt.

Ich besitze keinen entspechenden Doktorgrad und habe mich anderwärts nicht um einen Doktorgrad beworben.

Die dem Promotionsverfahren zugrunde liegende Promotionsordnung ist mir bekannt.

Berlin den ..... Shashank Gupta